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CEREAL CHEMISTRY

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CONTENTS

EDITORIAL POLICY

Cereal Chemistry publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

Cereal Chemistry gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

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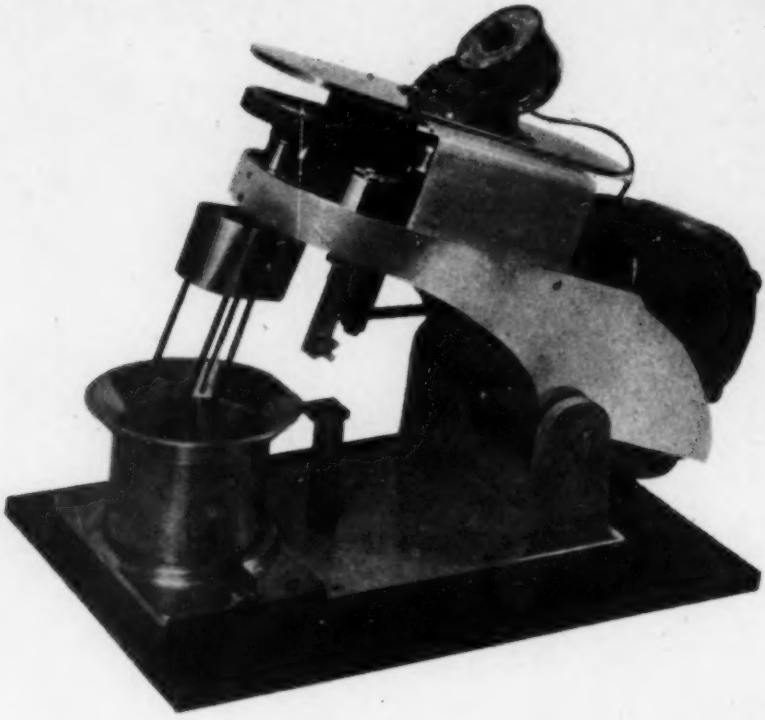

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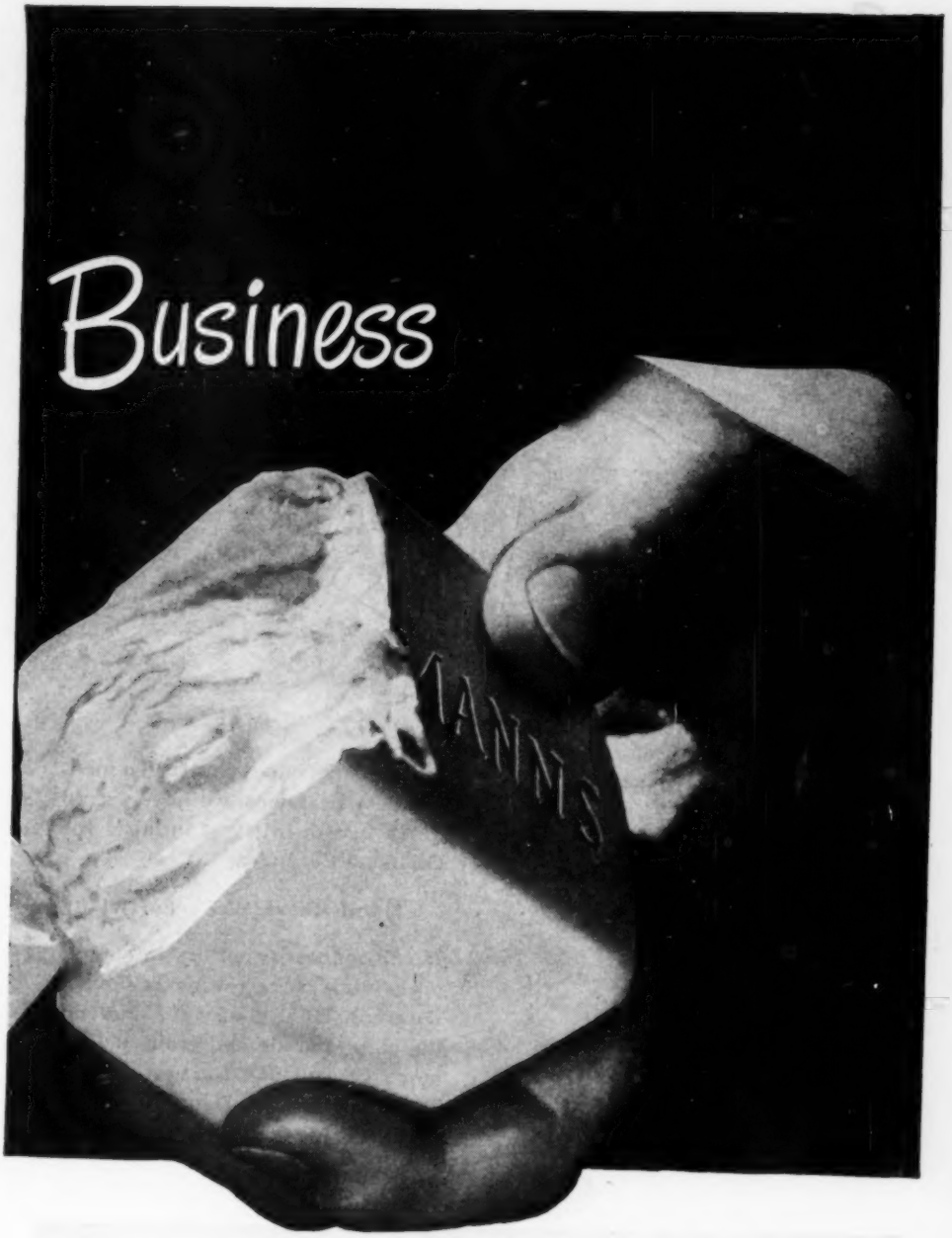

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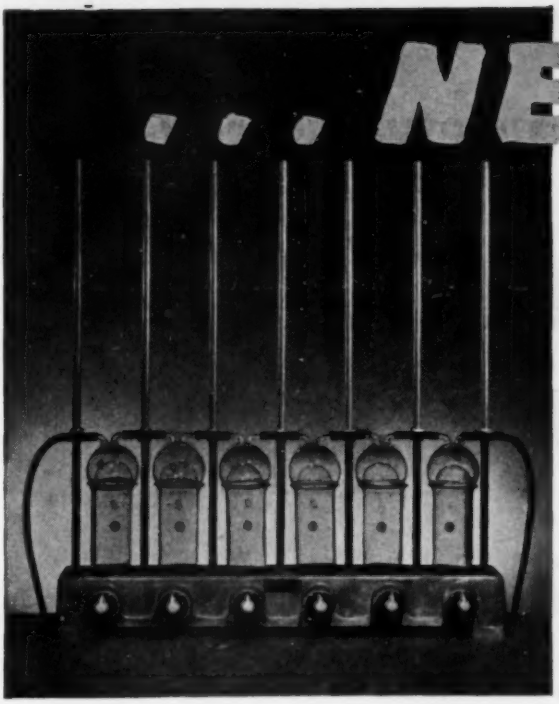

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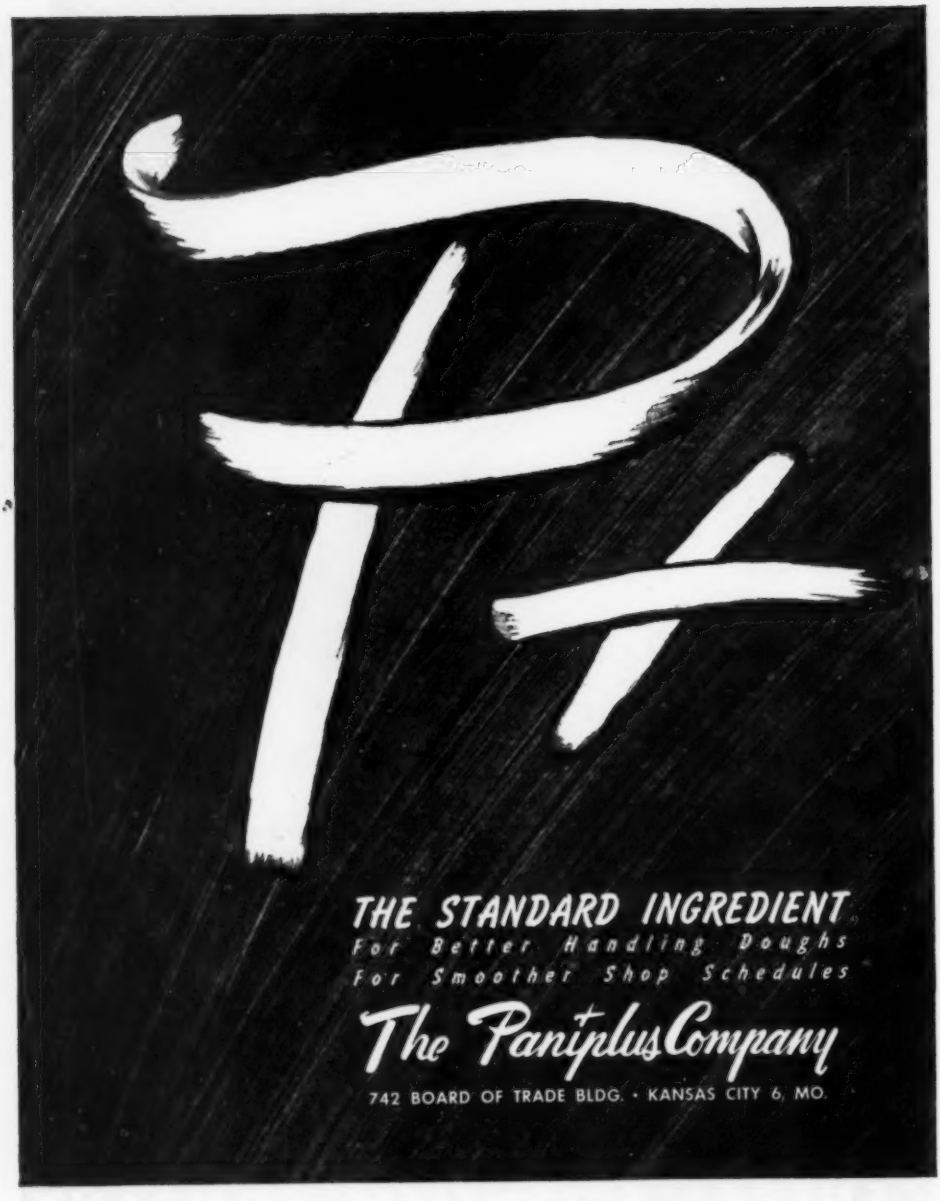
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CEREAL CHEMISTRY

VOL. XXV MAY, 1948 No. 3

METHODS FOR DETERMINING FLOUR PARTICLE SIZE DISTRIBUTION [1]

FRANK W. WICHSER and J. A. SHELLENBERGER [2]

ABSTRACT

The determination of the particle size distribution of wheat flour by sieving, air flotation, and sedimentation procedures is discussed. Data produced by a sieving procedure using Tyler wire screens and the Ro-Tap shaker are consistently accurate, applicable to any particle size range above 37μ, and simple to run. Flour separation by air using the Roller Air Analyzer produces particle size distribution data below the size of 80μ. The air procedure is more rapid than the other methods. A sedimentation method using the Andreasen pipette only produced accurate data below the size of 50μ.

The importance of a satisfactory method for determining the particle size distribution of flour was recognized long ago. Flour particles vary in size and exhibit marked differences in chemical and physical characteristics. Baking characteristics are partially influenced by the granularity of a flour.

Numerous investigations employing various methods have been conducted for determining flour particle size. While certain of these methods give reproducible results under the conditions of the test, they do not indicate the true particle size distribution. In developing reliable and accurate procedures it is necessary to compare data obtained by different methods with the same flour under equivalent standard conditions.

The method used extensively by previous investigators was one employing silk bolting cloths in a stack arrangement. The limitation of mesh fineness of the silk cloth and inaccuracies in the shape and size of the aperture openings presented serious drawbacks. Other limitations resulted from flour agglomerates, inadequate shaking devices, and the stack sieve arrangement.

A classification of the many possible methods for determining

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Contribution No. 147, Department of Milling Industry, Kansas State College, Manhattan, Kansas.
This research was supported by a grant from the Millers' National Federation.
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particle size distribution based on that of Scheyer and Work (10) is given below. Some of these methods may not be applicable to flour and others have been omitted because of insufficient data to justify a comparison with commonly used methods.

CLASSIFICATION OF METHODS FOR DETERMINING SIZE AND DISTRIBUTION OF PARTICLES

I. Sieve Analysis
II. Microscopic Analysis
  A. Conventional microscopic methods
  B. Ultramicroscopic methods
III. Sedimentation Analysis
  A. Increment methods:
    1. Pipette method
    2. Hydrometer method
    3. Pressure method
    4. Photographic method
  B. Cumulative methods:
    1. Balance methods
    2. Pressure methods
IV. Centrifugal Analysis
  A. Ordinary centrifuge methods
  B. Supercentrifuge methods
  C. Ultracentrifuge methods
V. Elutriation Analysis
  A. Air elutriation
  B. Liquid elutriation
VI. Turbidimetric Analysis
  A. Gross methods
  B. Size distribution methods
VII. Miscellaneous Methods
  A. Permeability methods
  B. Adsorption methods

The purpose of this investigation was to develop methods of wire sieve analysis, air elutriation, and sedimentation analysis, and to compare the results to determine the validity of each method for particle size distribution analysis of flour.

Materials and Methods

Commercially milled hard red spring, straight grade wheat flour was used, employing the one-half height W. S. Tyler standard screen scale sieves Nos. 100, 115, 150, 170, 200, 250, 270, 325, and 400 with the Tyler Ro-Tap shaker, the Roller Particle Size Air Analyzer, and the Andreasen pipette sedimentation column.

Sieve Analysis. The sieve analysis is a simple, accurate method of fractionating flour, but it is of little value unless made with a sieve having uniform square mesh openings. As silk bolting cloth has neither a uniform square mesh nor a sufficiently fine mesh to give accurate results, this precludes its use for flour granulation studies.

The Tyler Standard Screen Scale Testing Sieves, with aperture openings in the fixed ratio of the square, or fourth, root of two (1.414 and 1.189 respectively), fulfill the requirements for an accurate mesh sieve and are widely used for accurate particle size analysis. The sieves, used with the Ro-Tap shaker, produce dependable results if a

TABLE I

SIEVING DATA OBTAINED USING TYLER WIRE SCREENS AND RO-TAP SHAKER

Sieve mesh	Sifting time, min.	Material passing the sieve		Sieve mesh	Sifting time, min.	Material passing the sieve	
		I	II			I	II
		%	%			%	%
400	1	7.6	5.4	200	1	34.2	17.6
	2	12.4	9.6		2	47.0	29.2
	3	15.6	12.4		3	52.4	36.4
	4	17.6	14.8		4	55.0	42.8
	5	19.0	16.4		5	56.8	45.2
	6	20.0[1]	17.6		6	58.4[1]	47.2
	7	20.8	18.4[1]		7	59.2	48.8
	8	21.4	19.2		8	60.2	50.8
					9	60.8	52.4
325	1	5.6	5.2		10	61.4	53.6
	2	9.8	9.8		11		54.8
	3	13.8	13.8		12		56.4[1]
	4	17.2	17.2		13		57.2
	5	19.6	20.0				
	6	22.0	22.4	170	1	42.8	40.8
	7	23.8	24.0		2	57.4	54.4
	8	25.0	25.6		3	63.4	60.4
	9	26.2	26.4		4	66.8	63.6
	10	27.0[1]	27.6[1]		5	69.0	66.0
	11	27.8	28.4		6	70.4	67.8
					7	71.6	69.2
270	1	17.0	14.6		8	72.8[1]	70.4
	2	23.6	23.8		9	73.6	71.6
	3	27.0	28.6		10	74.4	72.6[1]
	4	29.4	31.0		11		73.4
	5	30.8	32.8				
	6	32.0	34.8	150	1	68.8	65.8
	7	33.2[1]	35.2[1]		2	77.8	75.4
	8	34.0	36.0		3	81.2	78.8
					4	83.0	80.8
250	1	19.4	12.2		5	84.4	82.6
	2	28.8	19.4		6	85.6[1]	83.6
	3	34.4	24.4		7	86.4	84.6[1]
	4	37.8	29.8		8		85.2
	5	40.4	33.4				
	6	42.0	36.2	115	1	87.0	84.2
	7	43.2	38.6		2	92.2	91.2
	8	44.4	40.4		3	93.8	93.8
	9	45.6	41.8		4	95.0[1]	94.8
	10	46.6[1]	43.8		5	95.6	95.8[1]
	11	47.4	44.2		6		96.4
	12	48.0	45.2	100	1	95.4	94.0
	13		46.2[1]		2	98.0[1]	97.4
	14		47.0		3	98.6	98.4[1]

[1] Optimal per cent of material passing sieve.

satisfactory procedure, such as that described by Wichser, Shellenberger, and Pence (13), is employed.

Data obtained using the above-named procedure for fractionating the hard red spring flour are presented in Table I. A footnote 1 indicates the point where the optimal per cent of material of each size has passed through the sieve. Additional material removed by continued sieving was probably the result of attrition and this amounts to approximately 0.8 per cent or less per minute. The number of minutes required to reach the end point varies with sieving conditions;

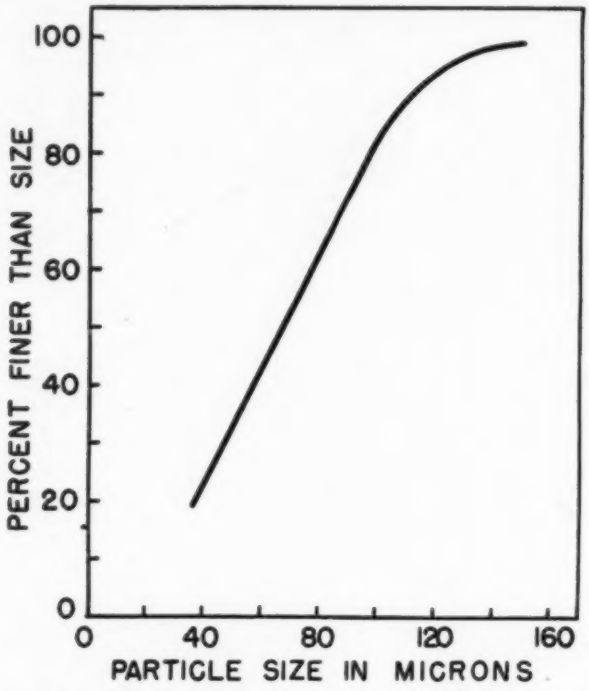

Fig. 1. Particle size distribution curve for wheat flour obtained by sieving.

however, the optimal per cent of material passing the sieve remains approximately the same for duplicate runs. A summation of the end point for all of the sieves establishes the particle size distribution curve illustrated in Fig. 1.

There are many advantages in the graphic method of showing the data obtained in screen analysis. Of the several methods of plotting these curves, the cumulative direct plot is most valuable and generally used. It is easy with a cumulative direct plot to find percentages that will pass, or be retained by, openings other than those used in the sieve test, and, conversely, to find the opening required to pass or re-

tain a designated percentage. Fig. 2 shows the data from Table I plotted by the cumulative direct plot method in the ratio of the fourth root of two (12). Particle size distribution curves for a straight grade winter wheat flour, and a straight grade soft wheat flour are also shown.

Air Elutriation. The Roller Particle Size Air Analyzer, described by Wichser, Shellenberger, and Pence (13), is a laboratory instrument used as standard equipment in other dry powdered industries (8) for determining particle size distribution. The instrument was designed to apply Stokes' Law to the separation of a powder mass of uniform

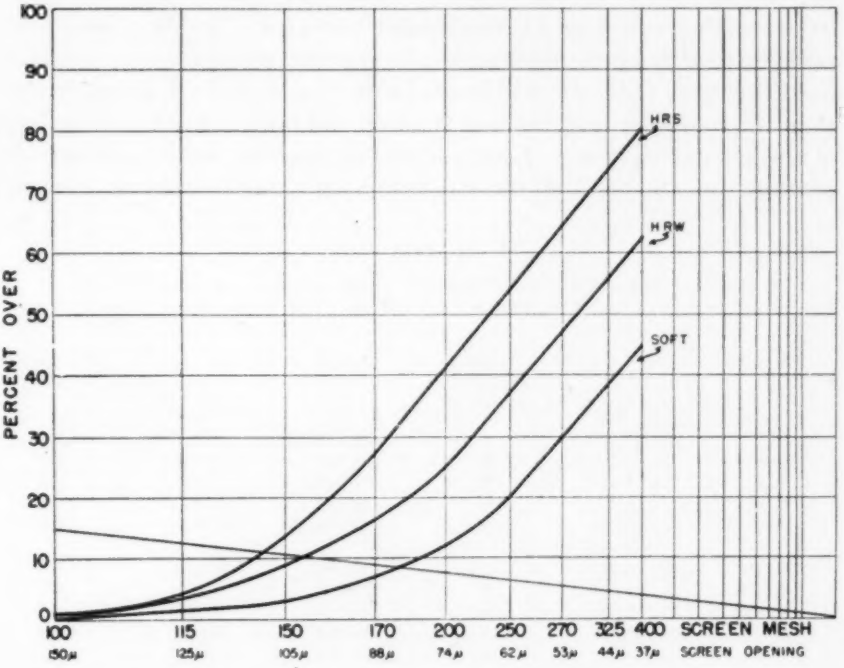

Fig. 2. Cumulative direct plot method for showing particle size distribution curves for flour.

density, but heterogeneous particle size, into fractions of a more uniform particle size. Stokes' Law relates the terminal velocity of fall:

$$V = \frac{10^{-8} g\, D\, d^2}{18n}$$

where V = terminal velocity of fall in cm./sec. in a stationary fluid,
g = constant of gravitation in cg. units = 980,
D = density of particle in g./ml.,
n = viscosity of fluid in cg. units = 1.82×10^{-4} for air,
d = diameter of particle in microns;

substituting in the above equation gives:

$$V = 0.00299\ Dd^2 \text{ cm./sec.}$$

With a knowledge of the true density of the powder, D, an air velocity is adjusted corresponding to the settling velocity of the coarsest particle in the finest fraction to be obtained. The particles finer than the arbitrarily selected coarse limit of the fraction are floated up through an expansion chamber and are filtered from the air. Oversized particles and agglomerates fall back into the U-tube, where the agglomerates are acted on by the jet of air and in the course of time are freed of their content of undersized particles. An increase in air velocity to that corresponding to the coarsest particle in the next desired fraction (Table II) will result in the removal of a fraction coarser than the coarse limit of the first fraction and finer than the coarse limit of the second fraction. Each succeeding increase in air velocity, depending on the size of the expansion chamber, removes a coarser

TABLE II

ROLLER AIR ANALYZER DATA FOR ADJUSTMENTS TO REMOVE THE DESIGNATED FLOUR-FRACTION SIZES

Fraction size	Air velocity	Cylinder diameter	Nozzle orifice	Capillary orifice	Flowmeter setting
μ	*L./min.*	*in.*	*in.*	*in.*	*in.*
0–5	2.60	9	0.038	0.067	2.6
0–6	3.72	9	0.042	0.067	4.9
0–7	4.82	9	0.046	0.067	7.7
0–8	6.45	9	0.055	0.067	13.6
0–9	8.10	9	0.059	0.067	19.7
0–10	10.40	9	0.070	0.120	2.8
0–12	14.88	9	0.082	0.120	5.4
0–14	19.28	9	0.096	0.120	8.8
0–16	25.80	9	0.104	0.120	15.0
0–18	8.10	4½	0.059	0.067	19.7
0–20	10.40	4½	0.070	0.120	2.8
0–24	14.88	4½	0.082	0.120	5.4
0–28	19.28	4½	0.096	0.120	8.8
0–32	25.80	4½	0.104	0.120	15.0
0–36	8.10	2¼	0.059	0.067	19.7
0–40	10.40	2¼	0.070	0.120	2.8
0–48	14.88	2¼	0.082	0.120	5.4
0–56	19.28	2¼	0.096	0.120	8.8
0–64	25.80	2¼	0.104	0.120	15.0
0–72	8.10	1⅛	0.059	0.067	19.7
0–80	10.40	1⅛	0.070	0.120	2.8
0–88	14.88	1⅛	0.082	0.120	5.4
0–96	19.28	1⅛	0.096	0.120	8.8
0–104	25.80	1⅛	0.104	0.120	15.0

fraction from the sample until a complete sample fractionation is accomplished.

A 5-g. sample of flour was introduced into the U-shaped sample tube. An air velocity, adjusted by a flowmeter, was such that it would remove a predetermined size fraction. The separation end point was determined by calculating the rate of separation. The initial rate was taken as that amount of material removed in a 10-minute interval. When the rate of separation was one-tenth of the initial rate, the operation was considered to have reached the end point. Beyond this latter rate was the rate at which slightly oversized particles appeared due to the inception of a parabolic velocity gradient. Succeeding trials established end points of separation for various other size fractions. The weight of each fraction removed is calculated as per cent of the original sample weight. Table III gives the data for the hard red

TABLE III

PARTICLE SIZE DISTRIBUTION DATA BY THE ROLLER AIR ANALYZER METHOD

Fraction size	Quantity of fraction separated I	II
μ	%	%
0–36	15.3	15.9
0–48	26.2	27.8
0–56	39.7	40.5
0–80	62.1	62.5
0–88	90.8	91.6
0–96	97.0	97.4

spring flour fractionated with the Roller Air Analyzer, with the particle size distribution curve shown in Fig. 3.

Pipette Sedimentation Method. The purpose of the present study was to attempt to select comparatively inexpensive simple sedimentation apparatus and to create conditions which would satisfy Stokes' Law so that the apparatus could be used for the entire particle size range of flour.

Sedimentation is the most frequently used process for the sizing analysis of particles normally below the size range of sieves. Many methods employing numerous types of equipment are described in the literature and have recently been reviewed by Stairmand (11), Heywood (4), Pieters and Hovers (9), and Davies (2).

It is necessary to begin sedimentation with a homogeneous mixture of flour particles and liquid medium. After a definite time lapse a variation in concentration of particles and medium will occur at a fixed depth below the surface. This concentration becomes a calculable function of particle size in Stokes' Law. The Andreasen pipette

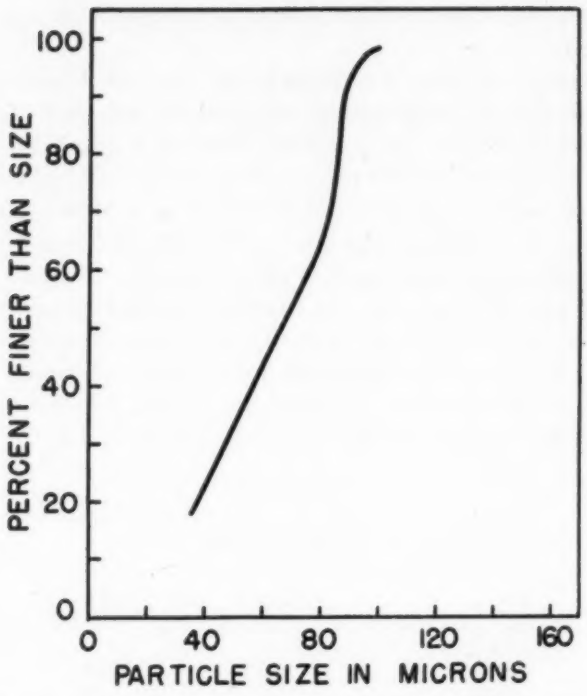

Fig. 3. Particle size distribution curve for wheat flour obtained by air separation.

apparatus was selected to determine this concentration by withdrawing small samples of the suspension. The Andreasen apparatus, shown in Fig. 4, consisted of a glass cylinder approximately 6 cm. in diameter with a capacity of 550 ml. when filled to the upper mark on the scale. It is provided with a ground glass stopper through which passes the stem of a pipette. The pipette extends 20 cm. beneath the surface of the suspension and 4 cm. from the cylinder bottom. The tip is at the level of the zero mark on the scale, while the upper surface of the suspension is at the 20 cm. mark. The pipette has a capacity of 10 ml. and is provided with a three-way stopcock and spout for draining into an evaporating dish. A uniform suction for withdrawing samples is provided by a water gravity-flow bottle.

Andreasen (1) found that Stokes' Law could be applied to angular or cubical particles of the same weight as spherical particles. By calculating the particle size as the edge length of a cube of the same volume as a sphere of radius r, his particle size conformed to the results of sieve analysis. This expression is shown as:

$$r = \left[2739 \sqrt{\frac{nh}{(D - d)tg}} \right] 1.612 \qquad \text{(A)}$$

where r = edge length of particle in microns,
n = viscosity of suspending medium in poises,
h = height in cm. between liquid surface and pipette tip when sample is drawn,
g = gravitation constant (980.3 dynes),
t = time in minutes of settling,
D = density of flour,
d = density of suspending medium.

The suspending medium was a mixture of carbon tetrachloride and naphtha. Viscosity was determined by a Ubellhode viscosimeter. The specific gravity of the suspending medium was determined by the usual method with a pycnometer. Air buoyancy corrections were applied to all weights. Flour specific gravity was determined by the pycnometric method using naphtha as the required medium.

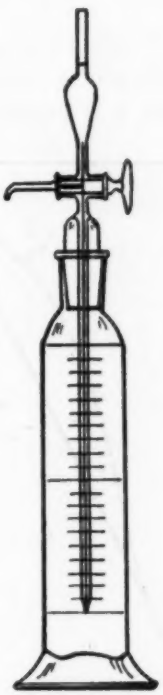

Fig. 4. Andreasen pipette sedimentation apparatus.

With the above values substituted into equation (A), the expression reduces to

$$t = \frac{k^2 h}{r^2}$$

where k is a constant for all the known values. The particle size (r)

TABLE IV

PARTICLE SIZE DISTRIBUTION DATA [1] FOR FLOUR BY THE ANDREASEN SEDIMENTATION METHOD

Calculated fraction size	Height of fall (h)	Time of fall (t)	I		II	
			Residue recovered	Finer than size	Residue recovered	Finer than size
μ	*cm.*	*min.*	*mg.*	%	*mg.*	%
Sample	20.4	0	99.8	100.0	100.3	100.0
125	20.0	1.50	94.2	94.4	96.5	96.0
100	19.6	2.30	80.0	80.1	92.9	92.5
80	19.2	3.52	61.5	61.6	81.1	80.7
65	18.8	5.22	38.8	38.9	63.3	63.0
50	18.4	8.64	—	—	32.7	32.5
40	18.0	13.20	21.0	21.1	21.3	21.2
30	17.6	23.00	14.7	14.7	17.2	17.1
20	17.2	50.50	10.4	10.4	10.3	10.2

[1] Specific gravity of suspending medium = 1.309, flour = 1.438.

is chosen, the height of fall (h) is determined, and the time for the rate of fall for the particle size selected is calculated.

A 6-g. portion of the flour was introduced into the column. The

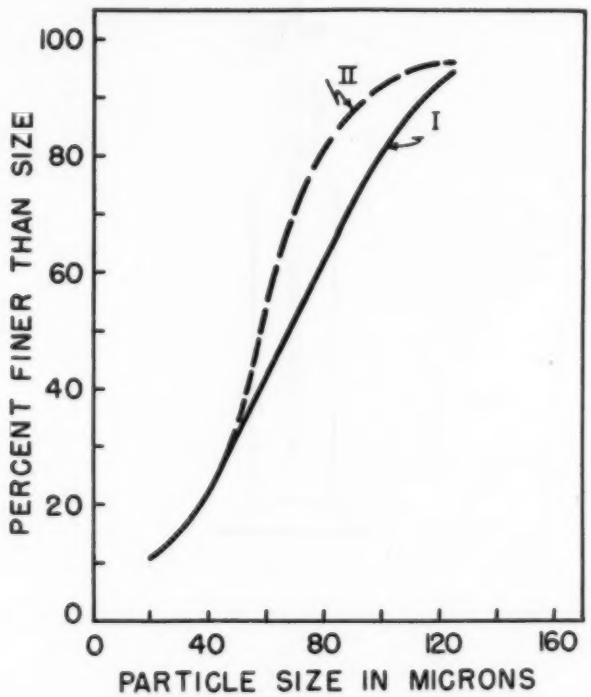

Fig. 5. Sedimentation curves obtained in duplicate trials with wheat flour illustrating the data in Table IV.

flour and medium were mixed thoroughly and an initial sample taken immediately. The apparatus was then placed in a constant temperature bath (30°C. ± 0.1°) and subsequent samples withdrawn at predetermined time intervals. Following discharge of the sample into a tared evaporating dish, the sample was dried and weighed. The data for a sedimentation trial on the hard red spring flour are given in Table IV, with the particle size distribution curves shown in Fig. 5.

Discussion

The means of data for sieving, air, and sedimentation analyses respectively are compared by the particle size distribution curves in Fig. 6.

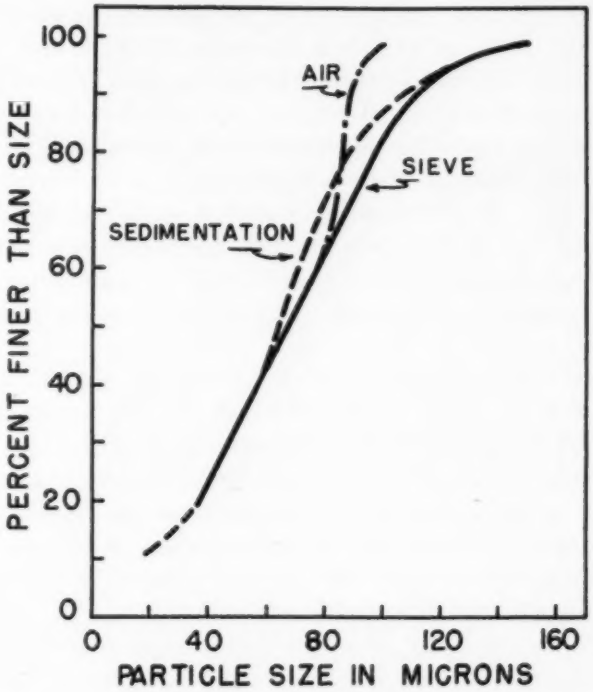

Fig. 6. Comparison of particle size distribution curves for wheat flour by sieving, air separation, and sedimentation.

The sieving test produces an accurately located curve for the entire size range of flour. Of the three methods, the apparatus required is the most applicable to the study of flour. The initial expense is moderate for the Ro-Tap and Tyler screens and the test requires less tedium for accurate data. For control work, it is not necessary to use all of the screens named. Four well-chosen screens are sufficient to produce data for an accurate particle size distribution curve. Many flours

were examined by the above method, with satisfactory results. The accuracy of the data is determined by a comparison with the results of other methods within their size range.

A stack sieve arrangement would shorten the sieving procedure somewhat if such a procedure would produce satisfactory data. Numerous attempts employing various sieve arrangements and cleaners were made. In no case were the data as reproducible as with the single sieve method. The variations in the curve characteristics by the stack arrangement were greater than the average differences of a vitreous and semivitreous wheat flour, thereby eliminating this arrangement as an accurate method of determining flour granulation.

The use of air for determining the particle size distribution in flour is an accurate method, but limited to the smaller size ranges. Fig. 6 shows that the air curve is in close agreement with the curve produced by sieving below the size of 80μ; for larger particles Stokes' Law is not valid using the Roller Air Analyzer. In addition to the factor of viscous resistance embodied in Stokes' Law, there is an added resistance due to the changes in momentum of the air in contact with the falling particles. A correction to Stokes' Law is possible (Gonell, 3) by modifying the air flow or correcting the approximate air flow to account for the resistance due to the change in momentum of the air, making size separation with the particle size analyzer applicable up to 150μ.

The theory of particle size separation by air flow is based upon spherical particles. Flour particles, except starch granules, are angular and substantially spherical, deviating only slightly from Stokes' Law. Thus, theoretically and practically, air is a desirable medium for flour particle size separation, especially in the finer size ranges. The disadvantages of using the Roller apparatus are few besides the initial expense and limited size range. The method is rapid, making it possible to establish many accurate separation points.

The sedimentation data were selected at random among the many sets of similar data. Fig. 6 shows a close agreement of the sieving, air, and sedimentation data below the 50-μ range, although poor agreement above this size. Many attempts were made by employing media of different specific gravities and employing refined sample withdrawal technique to overcome the pipette disadvantage of the Andreasen apparatus, where short time intervals for sample withdrawal in the large size ranges are encountered. Large errors are produced when the specific gravity of the medium approaches that of flour.

The disadvantages of the sedimentation method using the Andreasen apparatus are numerous. Exact calibration to obtain the physical

constants, as well as precise attention to details, is necessary to determine flour particle size. Also the apparatus must be used in a controlled temperature bath. Under the conditions used in this work the method gave poor data above the 50-μ size range.

Some advantages are seen in the sedimentation method employing other types of apparatus. Accuracy is obtained from apparatus employing a photo cell and galvanometer to determine the concentration gradient (6). The cumulative balance method was used successfully by Markley (7) and Hildebrand (5).

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HIGH LEVELS OF ALPHA-AMYLASE IN BAKING. I. EVALUATION OF THE EFFECT OF ALPHA-AMYLASE FROM VARIOUS SOURCES [1]

JOHN A. JOHNSON and BYRON S. MILLER [2]

ABSTRACT

The use in the baking process of high levels of alpha-amylase from various sources has been studied together with means of measuring amylase activity.

Improved response to malt supplements by the addition of ball-milled starch to normal flour is possible without the detrimental effects on gas retention usually noted when flour itself is overground. The improvement was associated with saccharifying activity of the amylases during fermentation rather than the liquefying activity during the baking.

Fungal alpha-amylase was less effective than equivalent concentrations of malted wheat or barley alpha-amylase in reducing the maximum viscosity of gelatinized starch. This difference was associated with the relative temperatures of inactivation of the different alpha-amylases. Fungal alpha-amylase, however, was slightly more effective in increasing gas production and in yielding improvements in the bread denoted as malt response.

The recommendation for malt requirement of flour depends on the method used for its measurement.

The use of amylases in baking has been adequately reviewed recently by Kneen and Sandstedt (12) and by Geddes (5). Millers and bakers do not agree as to what constitutes satisfactory malt supplementation. Johnson, Shellenberger, and Swanson (7) showed that flours manufactured for a specific use exhibited wide variations in maltose value, gassing power, and maximum viscosity as measured by the amylograph. Since the different methods of evaluating alpha-amylase depend on various physical and chemical changes, it is not surprising that a desired supplementation level estimated by one method does not agree with the requirements when another method is used.

Recently the amylograph has been gaining steadily in popularity as a rapid means for controlling alpha-amylase supplementation. Selman and Sumner (15) contend that the amylograph results are more valuable than either maltose or gassing power determinations because results of starch liquefaction with the amylograph are more comparable with the actual baking process. Anker and Geddes (3), however,

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caution against a too liberal interpretation of maximum flour gelatinization viscosity as an index of alpha-amylase activity. Maximum viscosity was found by them to be affected by starch concentration and susceptibility, pH, and salts, in addition to alpha-amylase activity.

Alsberg and Griffing (1), Sherwood and Bailey (16), Karacsonyi and Bailey (9), Jones (8), and Bottomley (4) have shown that increased maltose and gassing power are associated with the extent of damage and rupture of starch granules. Selman and Sumner (15), on the other hand, show that overgrinding is not associated with a corresponding decrease in maximum viscosity of the gelatinized starch. It thus appears that the effect of malt supplementation and the influence of starch granule susceptibility thereon may depend upon the method of measurement.

Although one of the proposed standards of the regulations of the Federal Food, Drug, and Cosmetic Act lists as optional ingredients for bread, malt syrup, dried malt syrup, malted barley flour, and malted wheat flour, which are diastatically active, the use of alpha-amylase from sources other than wheat or barley merits consideration. It must be recognized, however, that the use of different sources of alpha-amylase presents special problems in measurement, due to the fact that the enzymes possess different properties as well as different thermal inactivation points (Kneen and Sandstedt, 12). Several investigators including Hollenbeck and Blish (6) and Olson, Burkhart, and Dickson (13) have shown that a single enzyme is responsible for both dextrinization and liquefaction of starch. This, however, does not exclude the possibility that the dextrinization and liquefaction methods for measuring alpha-amylase activity may give entirely different relative results when alpha-amylases from different sources are employed.

The present study was undertaken to investigate the utility in baking of high levels of alpha-amylase from malted wheat flour, malted barley, and mold bran, and to study different means of measuring their effects.

In addition, a study of the effect of excessive grinding on starch susceptibility and its relation to alpha-amylase response and control was included.

Materials and Methods

The principal flour used was a commercial straight grade, unbleached, unmalted sample, having a protein content of 12.5% and an ash content of 0.45%. This flour showed good malt response in baking. For the study of the effect of ball-milling on malt response, several flours of hard red winter wheat varieties were available. Each

of these flours was a blend of several smaller straight grade, experimentally milled samples. These blends had an average protein content of approximately 12.0% and an ash content of 0.45% (14% moisture basis).

The sources of alpha-amylase employed included a commercial malted wheat flour having an activity of 48 alpha-amylase units, a commercial malted barley, ground on a Hobart laboratory mill before using, and a fungal alpha-amylase material, which was a wheat bran cultured with *Aspergillus oryzae*.

A straight-dough procedure was employed in baking. Optimum mixing was determined by observing dough characteristics. A 3-hour fermentation at 30°C. and two intermediate punches with the National pup sheeter was used in all cases. Moulding was done with a Thompson laboratory moulder, the proof was for 55 minutes at 30°C., and the loaves were baked for 25 minutes at 425°F.

The baking formula was varied according to the requirements of the experiment as shown in the following:

Ingredient	*Grams*
Flour	100 (14% moisture basis)
Yeast	2.0
Salt	1.5
Sugar	3 to 6
Shortening	3.0
Milk	0 to 4
Potassium bromate	0.001 to 0.003
Alpha-amylase supplement	According to experiment
Water (0.2% calcium chloride solution)	As required

The malt supplements were prepared by extracting the enzymes from their sources with an 0.2% calcium chloride solution for one hour at 30°C. Kneen and Sandstedt (11) have demonstrated that such extracts are satisfactory means of malt supplementation and that this procedure lends itself to experimental manipulation of the enzyme.[3] Also, preliminary experiments with extracts of malted wheat flour gave results comparable to experiments with the original malted wheat flour. In studying the effect of varying enzyme concentration, a 1:10 extract of the enzyme source usually had sufficient alpha-amylase activity to permit making the desired dilutions with 0.2% calcium chloride solution.

The alpha-amylase activity of these extracts was determined by the modified Wohlgemuth dextrinization method, employing excess beta-amylase, as prescribed by Sandstedt, Kneen, and Blish (14).

The proper dilutions of the various sources of alpha-amylase were calculated to correspond to malted wheat flour as a reference. All

[3] It is a common practice of bakers to add yeast foods containing calcium.

extracts are thus expressed in terms of relative alpha-amylase activity, based on the activity of 0.25% malted wheat flour, considered as normal or 1X concentration.

The starch used in studies dealing with starch susceptibility to amylase was washed under tap water from dough of the same sample of flour used in the baking experiments. The starch was separated from the liquid by centrifugation, dried at room temperature on glass plates before a fan, and ball-milled for a period of 24 hours in a pebble mill, rotating approximately 80 r.p.m.

In experiments on the temperature of inactivation of alpha-amylase, the usual buffered salt solutions (sodium citrate-hydrochloric acid or dibasic sodium phosphate-citric acid) precipitated the calcium present in the enzyme extracts. A flour extract was therefore used as a buffer medium for carrying the amylase enzymes in these experiments. By this procedure it was possible to subject the amylases to an environment of pH similar to that in bread dough.

The buffer medium was made by suspending 130 g. of the commercial flour in 225 ml. of distilled water for one hour. The flour suspension was then centrifuged and the centrifugate heated to boiling to destroy all enzymes, to gelatinize the starch, and to coagulate the soluble proteins. Precipitated material was then removed by filtering through cotton. After cooling, 0.5 g. of diastase (Merck and Company) was added to digest the gelatinized starch. This solution, after standing for 2 hours, showed the absence of free starch by the iodine test. The solution was heated again to inactivate the added diastase. The solution had a pH of 5.6, was clear, but possessed a slight yellowish hue.

To determine the temperature of enzyme inactivation, equivalent amounts of the enzymes were added to 225 ml. of flour extract and the final volume made up to 450 ml. with water. The flour extract concentration was thus comparable to that of a flour-water suspension used for a normal amylograph curve. Sufficient calcium chloride was added to give a concentration of 0.2%. This was placed in the amylograph bowl. The temperature was increased at a uniform rate of 1.5°C. per minute. An aliquot of 5 ml. was withdrawn after each five degree rise in temperature and immediately transferred to test tubes standing in ice-water. Alpha-amylase activity was determined on these aliquots by the dextrinization method of Sandstedt, Kneen, and Blish (14).

The amylograph curves for flour were made according to the method described by Anker and Geddes (3) using 65 g. (14% moisture basis) of flour and 450 ml. of liquid. Gassing power tests were made according to *Cereal Laboratory Methods*, 4th ed. (2).

Results and Discussion

Effect of Added Ball-Milled Starch on Malt Response. Preliminary experiments with numerous flour samples showed little baking response to malt supplements whether a formula rich or lean in sugar was employed. Amylograph curves made with these flours gave maximum viscosities of 1,000 Brabender units or more, suggesting a low level of

TABLE I

EFFECT OF ADDED BALL-MILLED STARCH [1] ON RESPONSE TO ALPHA-AMYLASE FROM VARIOUS SOURCES IN BAKING

Enzyme source	Relative enzyme concentration[2]	Loaf volume cc.	Grain[3] %	Texture %	Crust color	Break and shred
COMMERCIALLY MILLED FLOUR						
Control	0	675	80–o	75	Pale	Fair
Malted wheat	4X	698	80–o	77	Medium brown	Good
Malted wheat	12X	728	80–o	77	Dark brown	Very good
Malted barley	4X	698	83–o	79	Medium brown	Good
Malted barley	12X	745	85–o	82	Dark brown	Very good
Fungal	4X	735	83–o	75	Dark brown	Very good
Fungal	12X	755	90–o	93	Dark brown	Very good
EXPERIMENTALLY MILLED FLOUR						
Control	0	645	75–o	75	Pale	Fair
Malted wheat	4X	660	75–o	75	Light brown	Good
Malted wheat	12X	710	75–o	77	Dark brown	Very good
Malted barley	4X	663	80–o	77	Medium brown	Good
Malted barley	12X	705	85–o	82	Medium brown	Very good
Fungal	4X	685	80–o	75	Medium brown	Good
Fungal	12X	713	82–o	90	Dark brown	Very good

[1] 10 g. ball-milled starch substituted for 10 g. flour.
[2] 1X is equivalent to 0.25% malted wheat flour (48 alpha-amylase units) based on flour weight.
[3] o = open.

inherent alpha-amylase activity or resistance to amylase attack. These samples, after ball-milling for 1, 2, 8, 12, and 24 hour periods, were baked with several increments of malted wheat flour. The data obtained corroborated those of Alsberg and Griffing (1) in that ball-milling even for a period as short as one hour was detrimental to baking quality. The absorption capacity was increased, the loaf volume was decreased, the grain and texture became open and harsh, and the crust color became a darker brown. These characteristics were accentuated

as time of ball-milling was increased up to 8 hours. No further effect appeared beyond this time.

To improve the amylolytic characteristics of the flour without affecting the colloidal and gas-retaining properties as a dough, starch extracted from the flour samples was ball-milled for a 24-hour period and 10 g. of this starch were substituted for 10 g. of the flour.[4] Representative data from the baking tests with various concentrations of malted wheat flour, malted barley, and fungal alpha-amylase are presented in Table I. Both the commercially and experimentally milled flour responded to alpha-amylase supplements with the addition of ball-milled starch. Loaf volume, grain texture, crust color, and external appearance improved with increments of alpha-amylase supplements as high as an equivalence of 12 times the normal malt dosage. There was, furthermore, no slackening or stickiness in the doughs even at these high levels of alpha-amylase supplementation. The beneficial effects of wheat and barley alpha-amylase were similar but somewhat less marked than that of fungal alpha-amylase.

The influence of ball-milled starch on the maximum viscosity of flour paste in the presence of alpha-amylase is shown in Table II.

TABLE II

INFLUENCE OF ADDED BALL-MILLED STARCH ON MAXIMUM VISCOSITY OF FLOUR PASTES IN PRESENCE OF ALPHA-AMYLASE

Flour	Ball-milled starch	Maximum viscosity	
		No malt supplement	0.25% malted wheat flour
g.	*g.*	*B.U.*[1]	*B.U.*[1]
65	0	875	290
60	5	880	260
55	10	870	255
50	15	880	250

[1] B.U. = Brabender units.

The addition of ball-milled starch had little effect on the maximum viscosity. It is significant, however, that the ball-milled starch did not tend to increase the maximum viscosity as might be expected if raw starch was added to the flour. Ball-milled starch in the flour slightly decreased the maximum viscosity in the presence of 0.25% malted wheat flour; however, the amount of this decrease was not so great as was expected from the baking results. These data corroborated those of Selman and Sumner (15).

It would appear from the data in Tables I and II that the presence

[4] It is recognized that some bakers have followed the practice of adding solubilized starch to increase effectiveness of malt products.

of injured starch granules would affect the recommendation of malt requirement based on methods used to measure saccharifying activity, but would have little effect on the recommendations if based on amylograph data. The addition of ball-milled starch to flour is beneficial to amylase activity, and no detrimental effects appeared in gas retention.

Influence of Concentration of Alpha-Amylase from Various Sources on Maximum Viscosity. The influence of equivalent alpha-amylase concentrations from different sources on the maximum flour paste viscosity is shown in Table III. Malted barley alpha-amylase appears

TABLE III

INFLUENCE OF ALPHA-AMYLASE CONCENTRATION AND SOURCES UPON MAXIMUM VISCOSITY OF GELATINIZED FLOUR PASTE

Relative enzyme concentration[1]	Maximum viscosity
	B.U.[2]
Control	1000
Malted wheat flour alpha-amylase	
1X	450
12X	120
24X	50
Malted barley alpha-amylase	
1X	275
12X	90
24X	50
Fungal alpha-amylase	
1X	880
12X	720
24X	550

[1] 1X is equivalent to 0.25% malted wheat flour.
[2] B.U. = Brabender units.

to be the most effective in reducing the maximum viscosity, being followed closely by malted wheat flour. The fungal alpha-amylase was much less effective.

Comparative thermo-stabilities of the alpha-amylase from the three sources, as determined by methods already described, are shown in Table IV, in terms of the per cent of alpha-amylase activity remaining when the indicated temperature has been reached in the amylograph. Barley alpha-amylase was most resistant to thermal inactivation, being closely followed by malted-wheat alpha-amylase. Fungal alpha-amylase was the most susceptible to inactivation by heat. These data are in agreement with the observations of Hollenbeck and Blish (6). Inactivation was a gradual process, as shown by the fact that activity decreased over a relatively wide range of temperature.

Since the most active period of liquefaction by alpha-amylase is in the temperature range of starch gelatinization, the maximum viscosity

TABLE IV

EFFECT OF TEMPERATURE UPON THE INACTIVATION OF ALPHA-AMYLASE FROM DIFFERENT SOURCES

Temperature	Malted wheat flour alpha-amylase activity remaining	Malted barley flour alpha-amylase activity remaining	Fungal alpha-amylase activity remaining
°C.	%	%	%
60	100	100	100
65	97.9	100	100
70	91.0	100	63.0
75	29.1	71.9	6.3
80	14.5	29.1	0.0

is controlled to a large extent by the temperature at which the particular enzyme is inactivated. Thus the higher the temperature of inactivation of the alpha-amylase, assuming equivalent concentration, the greater would be its activity as measured by the amylograph. The differences in the maximum viscosity for the three alpha-amylase sources employed in this study may therefore be explained by the differences in thermal inactivation.

Effect of Malted Wheat Flour and Fungal Alpha-Amylase upon Gassing Power. Data showing the gassing power obtained from various amounts of malted wheat flour and fungal alpha-amylase are shown in Table V. Above 12X concentration of malted wheat flour alpha-amylase there was no increase in gassing power, but with fungal alpha-amylase there was a slight increase up to 24X concentration. The

TABLE V

EFFECT OF MALTED WHEAT FLOUR AND FUNGAL ALPHA-AMYLASE UPON GASSING POWER

Relative enzyme concentration[1]	Hours		
	1	6	24
	mm.	mm.	mm.
Control	86	343	539
MALTED WHEAT FLOUR ALPHA-AMYLASE			
1X	78	489	755
12X	107	710	1212
24X	110	696	1207
FUNGAL ALPHA-AMYLASE			
1X	102	596	980
12X	103	687	1213
24X	109	724	1346

[1] 1X is equivalent to 0.25% malted wheat flour.

effect of fungal alpha-amylase on gassing power at 12X concentration was equal to, and at 24X concentration even greater than, that of malted wheat flour alpha-amylase. These effects are opposite to those measured with the amylograph and suggest that recommendations of the malt requirement of a flour would vary, depending on the method employed to measure malt response.

Effect of Malted Wheat Flour and Fungal Alpha-Amylase Concentration on Baking Results. The amylograph technics may simulate the effect which alpha-amylase might have upon the starch during the baking process, while gassing power is related to the amylase effects during the fermentation process. The baking test, however, is the final criterion of proper malt supplementation. The data in Table VI

TABLE VI

EFFECT OF MALTED WHEAT FLOUR AND FUNGAL ALPHA-AMYLASE CONCENTRATION ON BAKING RESULTS

Relative enzyme concentration[1]	Loaf volume	Grain	Texture
	cc.	%	%
0	715	80–o	80
MALTED WHEAT FLOUR ALPHA-AMYLASE			
1X	760	83–o	85
12X	830	85–o	88
24X	850	88–o	92
FUNGAL ALPHA-AMYLASE			
1X	760	82–o	85
12X	850	88–o	95
24X	880	85–o	92

[1] 1X is equivalent to 0.25% malted wheat flour.

show baking effects of malt supplements at high levels. The baking was done with a formula containing 6% sugar so that adequate yeast nutrition was provided without dependence on sugar formation by amylase action. Both malted wheat flour and fungal alpha-amylase increased the loaf volume and improved other characteristics of the bread.

It would be anticipated from the work of Kozmin (10) that the high concentrations of alpha-amylase employed in this study would cause excessively wet and sticky doughs and soggy bread crumb. From the amylograph results it might also be expected that excessive starch liquefaction would occur with 12X and 24X concentrations of malted wheat flour as compared with equivalent concentrations of

fungal alpha-amylase. There was, however, no evidence of stickiness of dough or a soggy bread crumb with either malted wheat flour or fungal alpha-amylase.[5]

From the baking data (Table VI) employing the straight dough procedure it would appear that the upper safe limit of malt supplementation could exceed 24 times the normal concentration of 0.25% malted wheat flour or equivalent amounts of alpha-amylase from fungal or barley sources. The safe maximum limit will, of necessity, vary with the thermo-stability of the alpha-amylase. It would appear that if the alpha-amylase source has an exceedingly high inactivation temperature, detrimental effects would be obtained with large dosages. The concentration and temperature of inactivation of the alpha-amylases employed in these studies were not high enough to observe any detrimental effects on baking, yet the concentrations were 24 times greater than normally used.

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[5] It is recognized that failure to obtain these adverse effects of high concentration of alpha-amylase may be due, in part, to the particular baking procedure employed. This will be discussed further in the second paper of this series.

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HIGH LEVELS OF ALPHA-AMYLASE IN BAKING. II. PROTEOLYSIS IN STRAIGHT AND SPONGE DOUGHS [1]

BYRON S. MILLER and JOHN A. JOHNSON [2]

ABSTRACT

The use of high levels of alpha-amylase in both the straight and sponge dough baking procedures was investigated. Malt supplements containing excessive amounts of proteolytic enzymes, such as an extract of a fungal bran preparation, caused soft and sticky doughs, inferior grain and texture, and extremely low loaf volume when the sponge procedure was used. No harmful effects were noted when using the straight dough procedure.

Removal of proteolytic enzymes from amylolytic preparations by adsorption on kaolin indicated that the detrimental action produced by high levels of certain malt supplements in baking was due to their inherent proteolytic capacity.

Sodium chloride acted as a proteolytic inhibitor, but potassium bromate had no inhibitory action. The addition of sodium chloride to the sponge in the sponge baking procedure permitted the use of high levels of malt supplements containing proteolytic enzymes with no harmful effects. The use of high concentrations of alpha-amylase in the sponge procedure as well as in the straight dough procedure was practicable and would permit the use of smaller sugar concentrations in the dough.

Detrimental effects of certain malts and malt supplements have frequently been encountered and bakers are reluctant to add more than a very small percentage to their doughs. The literature, as reviewed by Hildebrand and Burkert (6), presents two conflicting theories on the cause of this detrimental action. The older theory proposed by Ford and Guthrie (2) postulates that the detrimental action is due to high proteolytic activity. The later theory originally proposed by

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Kozmin (8) and amplified by other writers including Hildebrand and Burkert (6), Geddes, Hildebrand, and Anderson (3), and Stamberg and Bailey (13) suggests that the increase in dough mobility and stickiness with the addition of excessive dosages of malt flour is due to the alpha-amylase activity of such malts rather than to their proteolytic activity. Kozmin (8) contended that when the alpha-amylase activity was excessively high the defects in the crumb were due to the starch degradation, producing dextrins which possessed decreased water-retaining capacity.

Johnson and Miller (7) showed that high levels of alpha-amylase may be used in straight doughs without the serious detrimental effects observed by Kozmin (8) and others. Read and Haas (10) noted that the action of certain enzyme preparations was more marked with the sponge method than with the straight dough baking procedures.

The detrimental effects ascribed to the presence of proteases in malts by earlier workers naturally led to attempts to remove them. Tissue and Bailey (14) concluded that proteolytic enzymes are almost completely removed from malted wheat preparations by precipitation with safranine. Read and Haas (11), however, were unable to effect a strictly quantitative removal of the proteases by this procedure.

Young and Hartman (15) found that several adsorbents including bauxite, permutite, kaolin, alumina, and pumice would remove trypsin from the amylolytic and lipolytic enzyme systems of pancreatic juice. Hemmi and Inami (4, 5) found that kaolin could be used for partially separating protease from the amylase of takadiastase and pancreatin preparations.

The objective of the present investigation was to study the use of high levels of alpha-amylase in baking with particular attention being directed to differences in proteolysis in straight and sponge dough baking procedures. Means of inhibiting proteolysis in sponge doughs and procedures for differentially separating proteolytic enzymes from amylolytic enzymes were also investigated.

Materials and Methods

The flour used in this study was the commercial straight grade, unbleached, unmalted sample having a protein content of 12.5% and an ash content of 0.45% (14.0% moisture basis) (Johnson and Miller, 7). This flour showed a good malt response in baking.

Two sources of alpha-amylase, a malted wheat flour and a fungal bran preparation, were employed.

The straight dough baking procedure employed by Johnson and Miller (7) was followed in the present study. All loaves were baked in

duplicate. In the sponge baking method the following formula was used:

	Sponge		Dough
	g.		g.
Flour	70 (14% moisture basis)		30 (14% moisture basis)
Yeast	2		0
Sugar	0		4
Salt	1.5	or	1.5
Milk	—		4
Shortening	—		3
Potassium bromate	—		0.003
Alpha-amylase (added in extract form)	According to experiment		
Water (0.2% calcium chloride solution)	60% of total		40% of total

The sponge was mixed for 1½ minutes, given a 4-hour sponge time, 30-minute floor time, 20-minute rest before panning, 55-minute proof time, and baked for 25 minutes at 425°F. The fermentation temperature was 30°C. The dough mixing time was varied to obtain optimum dough development and handling properties. All loaves were baked in duplicate.

Enzyme extracts were used exclusively and were prepared by a one-hour extraction (30°C.) of the original alpha-amylase source with 0.2% calcium chloride solution. Dilutions were also made with 0.2% calcium chloride solution. Alpha-amylase determinations and adjustments were made according to the procedure of Sandstedt, Kneen, and Blish (12). Determinations of proteolytic activity were made using an Ayre-Anderson procedure as modified by Miller (9). Gassing power tests were made by the pressuremeter method described in *Cereal Laboratory Methods*, 4th ed. (1).

In both baking procedures the enzyme concentrations used ranged from 0 to 24 times the normal concentration of alpha-amylase provided by 0.250 g. of malted wheat flour (48 alpha-amylase units) per 100 g. of flour. In the text and tables the enzyme concentrations carry the notation 1X, 12X, or 24X to indicate that the amount of enzyme used is 1, 12, or 24 times the normal amount.

Results

Baking Effects of High Malt Levels in Straight and Sponge Doughs. Table I presents the data obtained from both straight dough and sponge procedures. All samples were baked on the same day. The one-hour difference in the straight and sponge dough procedures in no way accounts for differences in dough and loaf characteristics.

By the straight dough procedure the loaf volume and crumb characteristics improved with increasing alpha-amylase concentrations up

TABLE I

COMPARISON OF THE STRAIGHT AND SPONGE DOUGH BAKING PROCEDURES EMPLOYING VARIOUS LEVELS OF MALTED WHEAT FLOUR AND FUNGAL ALPHA-AMYLASE

Source of alpha-amylase	Relative[1] enzyme concentration	Sugar %	Loaf characteristics			
			Loaf volume ml.	Grain[2] %	Texture %	Break & shred[3]
STRAIGHT DOUGH PROCEDURE						
Wheat malt	0	6	750	85–o	90	VG
Wheat malt	1X	6	755	85–o	90	VG
Wheat malt	12X	6	825	88–o	93	VG
Wheat malt	24X	6	825	89–o	93	VG
Wheat malt	0	3	670	83–c	80	F
Wheat malt	1X	3	685	85–c	82	G
Wheat malt	12X	3	755	90–c	86	G
Wheat malt	24X	3	775	90–c	90	VG
Fungal	1X	6	720	85–c	86	G
Fungal	12X	6	850	90–o	90	VG
Fungal	24X	6	870	93–c	95	G
Fungal	1X	3	675	83–c	80	F
Fungal	12X	3	785	86–o	85	VG
Fungal	24X	3	810	89–o	90	VG
SPONGE DOUGH PROCEDURE						
Wheat malt	0	4	685	87–c	85	F
Wheat malt	1X	4	710	91–c	90	G
Wheat malt	12X	4	760	85–c	85	G
Wheat malt	24X	4	810	83–o	84	F
Fungal	1X	4	720	90–c	87	G
Fungal	12X	4	675	75–o	70	P
Fungal	24X	4	580	50–o	50	P

[1] 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.
[2] c = close, o = open.
[3] VG = very good, G = good, F = fair, P = poor.

to 24X. The volume obtained when using 6% sugar was appreciably larger than that obtained when only 3% sugar was used with comparable enzyme concentrations. The addition of either malted wheat flour at levels up to 6 g. per 100 g. flour (24X concentration of alpha-amylase) or a 0.2% calcium chloride extract of the same malt supplement containing an equivalent concentration of alpha-amylase produced doughs with similar handling properties and identical loaf volumes. The grain and texture and crumb color, however, were inferior when the malted wheat flour itself was used at 24X concentration.

By the sponge baking procedure the use of malted wheat flour gave increases in loaf volume with increasing alpha-amylase concentration, but the crumb properties were adversely affected. When fungal amy-

lase was used, the loaf volume and the crumb properties became inferior with increasing concentrations of fungal alpha-amylase. Likewise, the dough became sticky and exceedingly difficult to handle. The probable cause of the variation in results obtained with malted wheat flour and mold bran was realized when it was found that the fungal extract showed eight times the proteolytic activity of an equivalent amount of malted wheat flour extract based on alpha-amylase content.

Inhibition of Proteolysis in Sponge Doughs by Sodium Chloride. The striking difference noted between the loaves baked by the sponge and straight dough methods (Table I) was confirmed by further tests.

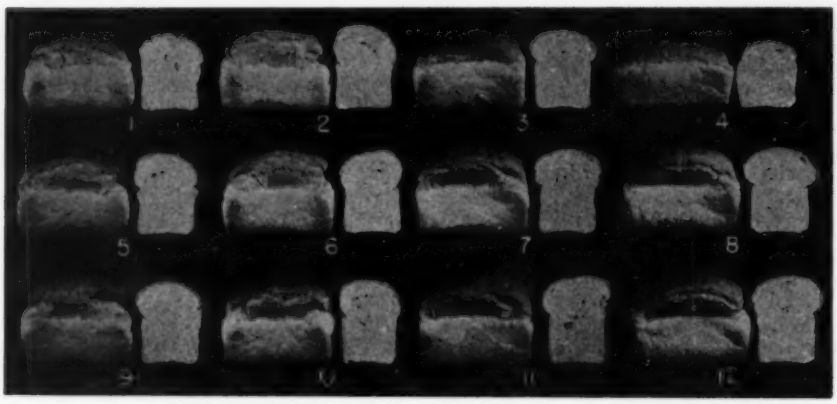

Fig. 1. Effect of sodium chloride in sponge doughs containing high levels of fungal alpha-amylase.

Alpha-amylase concentration	0	1X	12X	24X
No salt	1	2	3	4
1.05% salt	5	6	7	8
1.5% salt	9	10	11	12

Since only yeast and water normally are added to the sponge it was assumed that one or more of the baking ingredients normally added at the dough stage (straight dough procedure) prevented the appearance of the detrimental properties observed in the sponge.

Experiments were performed in which sodium chloride and potassium bromate were added both to the sponge and to the dough in the sponge baking procedure. The beneficial effects of adding sodium chloride to the sponge are shown by the baking data in Table II and by the photographs in Fig. 1. That potassium bromate did not restrain the detrimental action of high malt concentrations is shown in Table III and pictorially in Fig. 2.

Chemical data (Table IV) supporting the evidence provided by actual baking tests indicate that sodium chloride inhibits proteolytic enzymes while potassium bromate has no inhibitory effect. Table III and Fig. 2 also indicate that potassium bromate does not inhibit proteolytic activity during baking and that the optimum level of potassium bromate (3 mg. in this case) is independent of the concentration of alpha-amylase used or of the amount of proteolysis.

Removal of Proteolytic Enzymes. The data in Tables II and IV and Fig. 1 suggest that the detrimental effects observed in the sponge

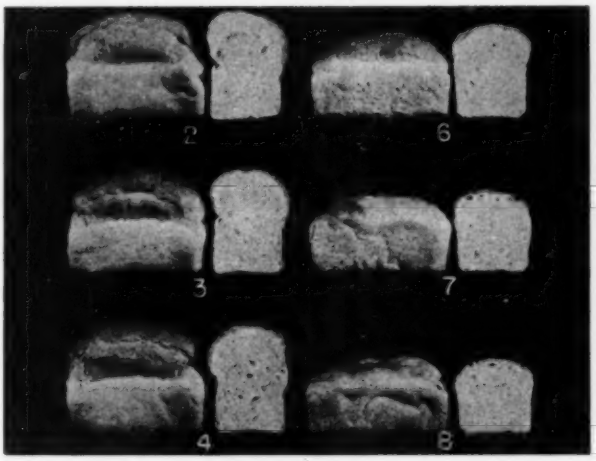

Fig. 2. Effect of potassium bromate in sponge doughs containing high levels of fungal alpha-amylase.

Alpha-amylase concentration	1X	24X
3 mg. % $KBrO_3$	2	6
10 mg. % $KBrO_3$	3	7
20 mg. % $KBrO_3$	4	8

method are due to excessive proteolysis. This view is further substantiated by the data in Table III and Fig. 2, which indicate that the detrimental effects of high malt concentrations are not associated with the presence of an excess of reducing matter which might have an effect similar to that produced by proteolytic enzymes on the physical dough characteristics. This is shown by the fact that high concentrations of potassium bromate failed to improve bread characteristics in the presence of excess fungal extracts. As determined by titration with 0.1 *N* standard iodine and 0.1 *N* sodium thiosulfate solutions, the malted wheat flour extract actually contained about four times the quantity of reducing matter as an equivalent concentration of fungal alpha-amylase.

TABLE II

EFFECT OF SODIUM CHLORIDE IN SPONGE DOUGHS CONTAINING HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Loaf no.	Relative enzyme concentration[1]	NaCl added to sponge	NaCl added to dough	Dough mixing time	Loaf characteristics				Dough handling at pan
					Volume	Grain[2]	Texture	Break & shred[3]	
		g.	*g.*	*min.*	*ml.*	%	%		
1	0	0	1.5	2	705	80–o	85	F	Good
2	1X	0	1.5	2	780	88–o	90	G	Good
3	12X	0	1.5	1	745	75–o	85	P	Slightly soft
4	24X	0	1.5	0.25	610	60–o	70	VP	Very soft
5	0	1.05	0.45	2	765	82–o	86	G	Good
6	1X	1.05	0.45	2	795	88–o	90	G	Good
7	12X	1.05	0.45	2	860	88–o	90	F	Good
8	24X	1.05	0.45	2	865	84–o	88	F	Slightly soft
9	0	1.5	0.0	2	770	82–o	85	G	Good
10	1X	1.5	0.0	2	785	86–o	88	G	Good
11	12X	1.5	0.0	2	850	86–o	90	F	Good
12	24X	1.5	0.0	1.75	860	83–o	88	F	Good

[1] 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.
[2] o = open.
[3] G = good, F = fair, P = poor, VP = very poor.

TABLE III

EFFECT OF POTASSIUM BROMATE ON SPONGE DOUGHS CONTAINING HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Loaf no.	Relative enzyme concentration[1]	$KBrO_3$ in sponge	Total $KBrO_3$	Dough mixing time	Loaf characteristics				Dough handling at pan
					Volume	Grain[3]	Texture	Break & shred[4]	
		mg.	*mg.*	*min.*	*ml.*	%	%		
1	1X	0	3	2	780	88–o	90	G	Normal
2	1X	3	3	2	860	85–o	88	VG	Normal
3	1X	10	10	1.75	815	82–o	85	VG	Normal
4	1X	20	20	1.75	795	78–o	80	G	Normal
5	24X	0	3	0.25	610	60–o	70	VP	Very soft
6	24X	3	3	0.25	650	70–o	70	VP	Very soft
7	24X	10	10	0.25	580[2]	60–o	60	VP	Very soft
8	24X	20	20	0.25	505[2]	50–o	40	VP	Very soft

[1] 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.
[2] Very yellow crumb color.
[3] o = open.
[4] G = good, VG = very good, VP = very poor.

Initial attempts to remove the proteolytic enzymes from amylase preparations were made using safranine dye and the procedure of Tissue and Bailey (14). This procedure was unsatisfactory, however, since the proteolytic activity could only be reduced 70% and a rather

TABLE IV

EFFECT OF SODIUM CHLORIDE AND POTASSIUM BROMATE ON PROTEOLYTIC ACTIVITY OF FUNGAL EXTRACT

NaCl	$KBrO_3$	Fungal extract	Nitrogen/10 mg. extract	Inhibition
mg.	*mg.*	*mg.*	*mg.*	*%*
—	—	25	9.4	0
85[1]	—	25	5.3	54
—	5.7[1]	25	9.4	0

[1] The quantities of salts used are in proportion to the amount used in baking.

large proportion of the alpha-amylase was simultaneously removed from the preparation.

To demonstrate more clearly the phenomena observed in the sponge procedure a method was developed to separate the proteolytic enzymes from alpha-amylase. A 25-ml. portion of fungal extract (1/10 dilution of the original extract) was added to kaolin (pH 7.3) and stirred periodically during a 30-minute standing time. The suspension was filtered under reduced pressure and the filtrate was checked for proteolytic and

TABLE V

REMOVAL OF PROTEOLYTIC ENZYMES FROM A FUNGAL AMYLOLYTIC PREPARATION USING KAOLIN AS AN ADSORBENT

Weight of kaolin[1]	Reaction	Enzyme activity of filtrate[2]	
		Amylolytic activity retained	Proteolytic activity retained
g.	*pH*	*%*	*%*
0	4.7	100	100
0.25	4.7	100	95
0.50	4.7	100	88
1.0	4.7	93	70
2.0	4.7	60	25
2.0	7.3	60	6

[1] Added to a 25-ml. portion of fungal extract (30 minutes contact time).
[2] The equivalent of 1.5 mg. mold bran used for the amylolytic determination and 10 mg. for the proteolytic determination.

amylolytic activity. The kaolin proved to be effective in removing proteolytic enzymes, but this was accompanied by adsorption of considerable alpha-amylase. The data in Table V show the effectiveness of kaolin for removing proteolytic enzymes from a fungal amylase preparation.

Baking data showing the effect of removal of proteolytic enzymes from a fungal alpha-amylase preparation by adsorption on kaolin (pH 7.3) are presented in Table VI. A photograph of the loaves is shown in Fig. 3.

TABLE VI

EFFECTS OF REMOVING PROTEOLYTIC ENZYMES FROM THE FUNGAL ALPHA-AMYLASE PREPARATIONS USED IN BOTH THE SPONGE AND STRAIGHT DOUGH BAKING PROCEDURES

Loaf no.	Relative enzyme concentration[1]	Proteolytic enzymes	Mixing time min.	Loaf characteristics				
				Volume ml.	Grain[2] %	Texture %	Crumb color %	Break & shred[3]
STRAIGHT DOUGH PROCEDURE								
1	0		3.0	800	80–o	83	86	G
2	1X	Present	3.0	785	83–o	85	90	G
3	12X	Present	3.0	825	88–c	87	90	VG
4	24X	Present	2.5	860	88–c	92	90	VG
5	1X	Removed	3.0	760	83–o	83	88	G
6	12X	Removed	3.0	825	83–o	83	88	VG
7	24X	Removed	3.0	850	86–o	85	90	VG
SPONGE DOUGH PROCEDURE								
8	0		2.0	745	80–o	83	86	G
9	1X	Present	2.0	790	80–o	83	86	G
10	12X	Present	1.0	750	75–o	78	82	P
11	24X	Present	0.5	660	60–o	70	70	VP
12	1X	Removed	2.0	785	88–o	85	88	G
13	12X	Removed	2.0	820	88–c	88	90	VG
14	24X	Removed	1.5	830	80–c	90	90	VG

[1] 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.
[2] o = open, c = close.
[3] G = good, VG = very good, P = poor, VP = very poor.

TABLE VII

EFFECT OF PROTEOLYTIC ENZYMES ON THE GAS-PRODUCING CAPACITY OF A FLOUR CONTAINING HIGH LEVELS OF FUNGAL ALPHA-AMYLASE

Relative enzyme concentration[1]	Proteolytic enzymes	Hours		
		1	6	24
		Manometric pressure		
		mm.	*mm.*	*mm.*
0		86	343	538
12X	Present	87	561	846
12X	Removed	90	568	857
24X	Present	90	614	989
24X	Removed	86	579	932

[1] 1X = normal concentration of alpha-amylase equivalent to that provided by 0.250 g. of malted wheat flour per 100 g. of flour.

The removal of proteolytic enzymes caused improvement in the loaf characteristics, mixing properties, and in loaf volume by both

the straight and sponge dough procedures. This improvement in the loaves baked by the sponge process with an enzyme preparation freed of proteolytic enzymes by adsorption is striking in comparison with the deterioration of the loaves baked by the same process with the original alpha-amylase preparation.

Effect of Proteolytic Enzymes on Gassing Power. The presence or absence of natural proteolytic enzymes in fungal amylase extracts

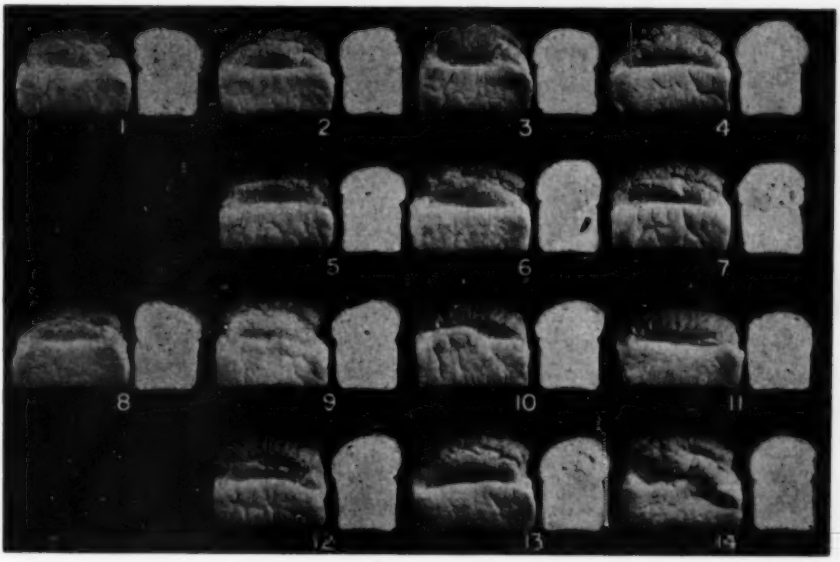

Fig. 3. Effects of removing proteolytic enzymes from the fungal alpha-amylase preparations used in both the sponge and straight dough baking procedures.

Alpha-amylase concentration	0	1X	12X	24X
Straight dough				
Proteolytic enzymes present	1	2	3	4
Proteolytic enzymes removed	—	5	6	7
Sponge dough				
Proteolytic enzymes present	8	9	10	11
Proteolytic enzymes removed	—	12	13	14

is shown by the data in Table VII to have no marked effect on the gas production capacity of the flour. Gas retention, on the other hand, was notably influenced as shown by reduction in loaf volume and the deterioration in bread characteristics recorded in Table VI. The effect of proteolytic enzymes on gluten also was evidenced by the stickiness of the doughs and by the very short re-mix times required for the sponge.

Discussion

The data clearly indicate the marked difference in the effect of proteolytic enzymes on the straight and sponge procedures. When high concentrations of alpha-amylase extracts containing large amounts of proteolytic enzymes were used in the sponge procedure, the re-mix time was reduced, the dough became extremely soft and sticky, the loaf volume was greatly decreased, and loaf and crumb characteristics became inferior. In the straight dough procedure, large concentrations of a similar preparation caused no harmful effects and even improved the quality of the bread.

Most authors suggest that the increase in mobility and stickiness resulting from large dosages of amylase preparations is due to excessive alpha-amylase activity rather than to proteolytic activity. The present work, however, indicates that malt extracts having high proteolytic activity are undesirable as baking adjuncts due to their effect on "gas retention capacity," as well as to their detrimental effects on physical dough properties. This appears to substantiate the idea of Ford and Guthrie (2).

The use of equivalent quantities of fungal and wheat malt alpha-amylase in the sponge procedure (Table I) reveals a sharp decline in loaf volume and crumb characteristics with increasing levels of fungal alpha-amylase. In contrast to this, the bread quality steadily improved with equivalent quantities of wheat malt alpha-amylase up to 12X. This difference in the action of the two supplements may be explained by the fact that the fungal preparation had eight times as much proteolytic activity as an equivalent amount of malted wheat flour alpha-amylase. When a large portion of the proteolytic activity was removed from the extract of fungal amylase by adsorption on kaolin, the baking results (Table VI) were similar to those obtained when malted wheat flour was used. An increase in loaf volume and an improvement in crumb properties were then obtained with increasing concentrations of the fungal alpha-amylase extract.

The data in Table II indicate that the addition of sodium chloride to the sponge tends to equalize the two baking procedures with respect to proteolytic activity. The deleterious effect of large additions of fungal amylase on loaf volume, handling, and crumb characteristics was counteracted by the addition of salt to the sponge. It made little difference whether the entire amount of sodium chloride was included in the sponge or whether a proportionate amount was added to the sponge and to the dough. This technic of adding a part of the salt to the sponge when the flour shows stickiness at the dough stage is a fairly common bakeshop practice.

The inhibition of fungal proteolytic enzymes by sodium chloride as determined by the modified Ayre-Anderson procedure (9) was found to be approximately 55% when using a concentration of salt equivalent to that present in the water added to the sponge dough. The data in Table III show that potassium bromate does not influence proteolytic action and that the optimum bromate level is 3 mg. whether 1X or 24X concentration of fungal amylase is used. Chemical determinations (Table IV) using the modified Ayre-Anderson procedure (9) confirm the failure of potassium bromate to influence proteolytic action. These data would appear to support the opinion that potassium bromate acts directly upon the gluten proteins and is not associated with the inhibition of proteolytic enzymes.

The possibility of using high levels of alpha-amylase to supplant, under emergency conditions, part of the sugar normally added in the straight dough procedure is suggested by the data in Table I. When either malted wheat flour or fungal alpha-amylase at 24X concentration was used in conjunction with only 3% sugar, the results were superior to those obtained from 1X concentration of enzyme and 6% sugar.

Further, the data in Table VI indicate that it is possible to use high levels of alpha-amylase in the normal sponge procedure when no salt is added to the sponge, provided the proteolytic enzymes are removed from the amylolytic preparation. Bread of high quality was produced by both straight dough and sponge procedures when using concentrations up to 24X of alpha-amylase, from which the proteolytic enzymes had been removed.

Acknowledgments

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ACTION OF MOLD ENZYMES IN STARCH SACCHARIFICATION [1]

JULIAN CORMAN and A. F. LANGLYKKE [2]

ABSTRACT

Alcohol yields from fermentation of corn mashes saccharified with mold culture filtrates correlated more closely with the potency of a glucogenic enzyme system, as measured by maltose hydrolysis, than with fungal alpha-amylase. This observation led to a detailed study of starch saccharification by fungal diastatic preparations with wide variations in the two types of enzyme systems.

Fungal alpha-amylase liquefies the starch and converts it to dextrins and maltose, while the glucogenic enzyme system hydrolyzes maltose, dextrins, and apparently starch itself to glucose. Starch saccharification efficiency showed a higher correlation with glucogenic activity than with alpha-amylase. High glucogenic activity was associated with rapid and almost quantitative glucose formation from starch. Starch degradation with a *Rhizopus* culture filtrate having only a trace of alpha-amylase activity showed production of considerable glucose but no maltose, although the blue-starch iodine reaction persisted.

Potent mold culture filtrates have low Lintner values, for they apparently contain little or no beta-amylase. Nevertheless, they saccharified starch to produce ultimate yields of fermentable sugars that were as high or higher than was obtained by saccharification with the distillers' malt sample used for reference.

In China and Japan starch saccharification by mold enzymes has been practiced for centuries. The Amylo process described by Grove (3) for commercial production of alcohol employed amylolytic fungi

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originating in those countries and was first applied in France. In this process the mold is grown directly in the grain mash which is saccharified by the fungal enzymes thus developed. Erb and Hildebrandt (2) and Underkofler *et al.* (11, 12) in this country have reported improved alcohol yields when fungal amylases were used commercially to saccharify grain mashes.

Le Mense, Corman, *et al.* (4) at this laboratory have recently reported a method for producing fungal amylases by submerged cultivation of fungi in distillers' thin stillage supplemented with 1% corn and 0.5% calcium carbonate. After 66 hours aeration, the culture liquors were used successfully by Le Mense, Sohns, and co-workers (5) in the pilot plant to replace completely the barley malt conventionally used in alcohol production. These culture filtrates have been used also by Efron and Blom (1) to make saccharine syrups from grain.

At first it was assumed that the major factor contributing to efficient starch conversion by fungal amylase preparations was alpha-amylase activity. Therefore, in a preliminary survey, mold strains were selected on the basis of their ability to produce high levels of alpha-amylase during submerged culture. However, it was soon found that the yields of alcohol on fermentation did not correlate with the alpha-amylase content of the culture filtrates. Subsequent studies indicated that rapid and complete saccharification might be related to the presence of a supplementary carbohydrase system which was measured by maltose hydrolysis. This "maltase" enzyme is subsequently referred to in this paper as "glucogenic activity." The enzyme or enzyme system involved appears to be capable also of attacking higher glucose polymers such as dextrins and even starch.[3]

Recognizing the presence of both alpha-amylase activity and glucogenic activity in our fungal amylase preparations, we investigated the correlation between levels of each type of enzyme activity and alcohol yields obtained from corn mashes saccharified with our mold culture filtrates. We also studied the progress of starch hydrolysis by fungal preparations varying widely in each type of enzyme activity and determined the nature of the starch degradation products obtained. Similar studies were made with an extract of distillers' malt for comparative purposes.

Experimental

Fungal amylase preparations were produced by submerged growth of molds in distillers' thin stillage supplemented with corn and calcium carbonate as described by Le Mense, Corman, *et al.* (4).

[3] In other papers on this subject from this laboratory (4, 5) and elsewhere the terms "maltase" and "maltase activity" are used. However, objection can be raised to these terms since the enzyme or enzyme system hydrolyzes higher glucose polymers as well as maltose. Accordingly in this paper, we have adopted the new term "glucogenic activity." These different terms in various papers refer, therefore, to the same thing.

Alcohol yields were determined by fermentation tests as follows: A slurry of 50 g. of ground corn and 200 ml. tap water in a 500-ml. Erlenmeyer flask was steamed at atmospheric pressure in the autoclave for 10 minutes. The hot mash was stirred well, cooked at 25 pounds steam pressure for 30 minutes, and then cooled to 72°C. Its temperature was further lowered to 55°–58°C. by vigorously stirring into it a mixture of 25 ml. of culture filtrate and 25 ml. tap water. The flask was placed in a 55°C. water bath for 30 minutes after which the mash was cooled to 30°C. and inoculated with 10 ml. of a 24-hour culture of *Saccharomyces cerevisiae* NRRL Y-567. After 72 hours' fermentation at 30°C. the entire mash was distilled until about 99 ml. was collected in a 100-ml. volumetric flask. The solution was diluted to volume with water and the alcohol yield determined with a dipping refractometer.

Alpha-amylase was determined by the Sandstedt, Kneen, and Blish procedure (8) as modified by Olson, Evans, and Dickson (6, 7).

The amount of alpha-amylase represented by one unit dextrinizes 1 g. of starch (pretreated with excess beta-amylase) in one hour at 20°C.

Glucogenic activity was determined by measuring the extent of conversion of maltose monohydrate to glucose when a reaction mixture containing two volumes of 1.05% maltose monohydrate and one volume of culture filtrate was incubated for two hours at 30°C. A pH of 4.6 was maintained in the reaction mixture by use of 0.137 M acetic acid-sodium acetate buffer. Glucogenic activity is expressed as the percentage of maltose monohydrate hydrolyzed to glucose under these conditions. The extent of hydrolysis was determined by the Somogyi (10) micro sugar method using the 20-minute heating time.

Progress of starch hydrolysis was followed by measuring the development of reducing power at various time intervals by the above-mentioned Somogyi method. Analysis for glucose, maltose, and dextrins in the presence of each other was made by the Somogyi (9) differential yeast fermentation procedure.

Alcohol Production. Alcohol yields obtained by use of some fungal amylase preparations for preliminary saccharification of the corn mashes are given in Table I. It is apparent that alcohol yields correlate more closely with glucogenic activity than with alpha-amylase activity. A high alcohol yield was obtained from the corn mash converted with the culture filtrate from *Aspergillus niger* NRRL 605 in spite of its unusually low alpha-amylase value. High glucogenic activity, however, was present in this preparation. On the other hand, saccharification with a culture filtrate of *Aspergillus foetidus* NRRL 341 with relatively good alpha-amylase value but low glucogenic activity was followed by poor fermentation.

The glucogenic activities of some fungal amylase preparations prepared from the same stock slant under essentially identical conditions sometimes vary. In Table I are listed two such preparations. These were inoculated on different days from the same stock slant of *Aspergillus oryzae* NRRL 464 to standard amylase producing media, and cultivated as usual. Although the alpha-amylase values of both preparations were approximately the same, glucogenic activities differed considerably. The higher alcohol yield was again associated with the preparation of high glucogenic activity, whereas a lower alcohol yield

TABLE I

ALCOHOL YIELDS FROM CORN MASHES SACCHARIFIED WITH FUNGAL AMYLASES

Culture	Glucogenic activity, % maltose hydrolyzed	Alpha-amylase, units per ml.	Ethanol,[1] proof gal. per bu.
A. niger 330	77.9	5.3	5.24
A. phoenicis 363	69.6	9.7	5.14
A. wentii 378	65.6	2.4	5.14
A. oryzae 464[2]	64.6	3.0	5.40
A. niger 605	61.7	0.1	5.05
A. niger 326	60.6	4.5	5.29
A. niger 354	47.6	3.7	4.97
A. wentii 382	28.5	1.5	4.97
A. wentii 377	25.4	0.9	4.44
A. niger 337	21.9	12.5	5.09
R. delamar 1705	19.6	trace[3]	4.58
R. sp. "Boulard" 1891	19.5	trace[3]	4.47
A. oryzae 694	12.2	8.3	4.85
A. oryzae 464[2]	11.5	2.9	4.71
A. foetidus 341	11.4	7.7	4.58

[1] Alcohol yield of 10% malt control was 5.1 proof gallons per bushel.
[2] Both culture filtrates inoculated from same stock slant.
[3] Culture filtrate assayed less than 0.08 alpha-amylase unit per ml.

resulted when fermentation followed the use of the preparation of low glucogenic activity.

Thus it appears that the supplementary carbohydrase system measured by maltose hydrolysis is an important factor in determining the efficiency of starch hydrolyzing fungal amylase preparations used for alcohol production.

Alpha-amylase, on the other hand, appears to be the main source of liquefying action. Conversion by culture filtrates of high alpha-amylase potencies resulted in very thin liquid mashes, while conversion with preparations of lower alpha-amylase activity produced smooth mashes which were not quite as liquid. Conversion with *Rhizopus* and *Mucor* culture filtrates invariably resulted in thick, gummy mashes, apparently because of the lack of alpha-amylase in these preparations.

Progress of Starch Hydrolysis by Mold Enzymes. In view of the common use of total reducing power determinations as a measure of starch saccharifying efficiency, the progress of starch hydrolysis was followed in terms of development of reducing power. Culture filtrates from four mold strains, as well as an extract of 180° Lintner barley malt, were used to saccharify 2% soluble starch. The reaction mix-

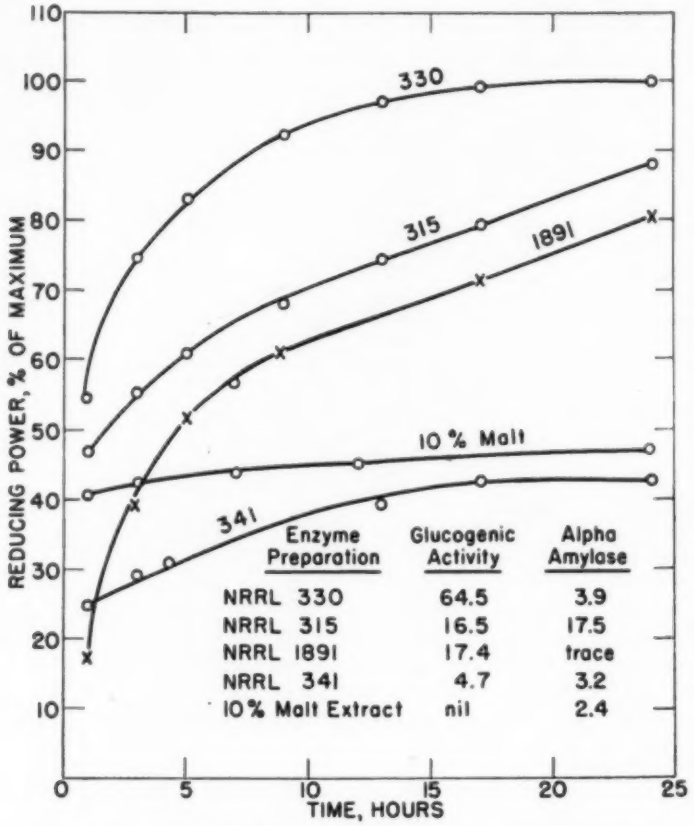

Fig. 1. Starch hydrolysis with various amylolytic preparations at 55°C., in terms of development of reducing power.

tures, consisting of 40 ml. of 2% soluble starch and 4 ml. of culture filtrate, or malt extract, were incubated at 55°C. and pH 4.6. "Maximum" reducing power was that developed by acid hydrolysis on a separate starch aliquot and thus corresponds approximately to the value that would be obtained if the starch were completely hydrolyzed to glucose.

Both the rate and extent of reducing power development increased as the glucogenic activities of the mold culture filtrates increased from

4.7 to 64.5, as shown in Fig. 1. No such relationship exists between fungal alpha-amylase activity and reducing power. These observations substantiate our conclusions from the fermentation studies that fungal alpha-amylase alone is not the determining factor in efficient

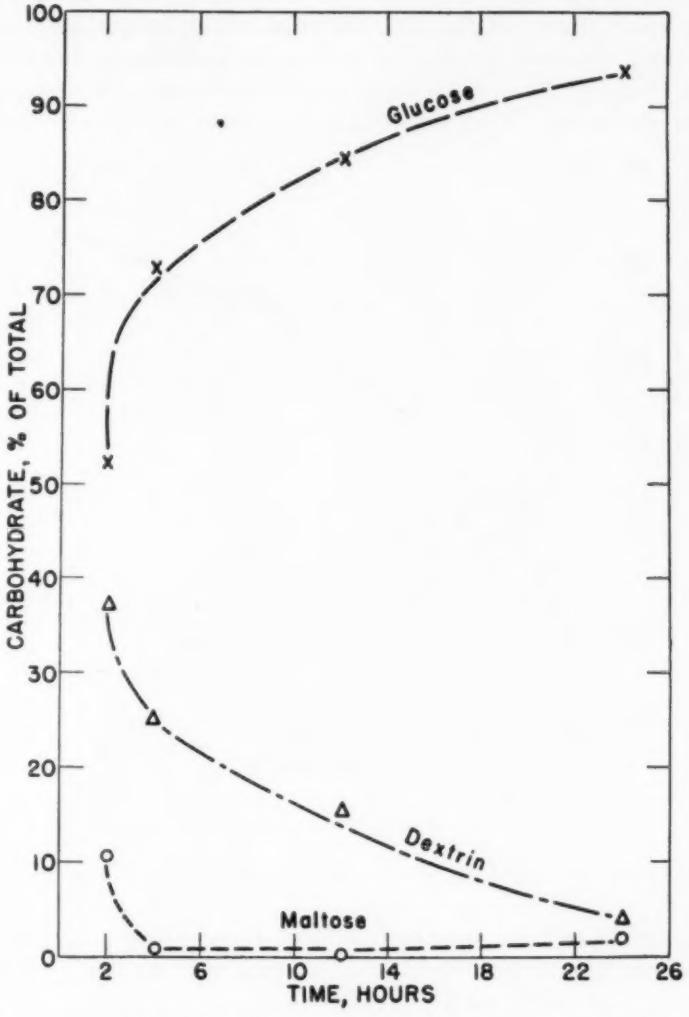

Fig. 2. Starch hydrolysis with *Aspergillus niger* NRRL 330 (at 55°C.) culture filtrate having 64.5 glucogenic activity and 3.9 alpha-amylase units per ml.

starch saccharification, but that a supplementary carbohydrase reflected by high glucogenic activity is also apparently necessary.

The difference in the rate of starch saccharification by mold enzymes from that by malt extract is also demonstrated in Fig. 1. In the case of malt hydrolysis, the reducing power attained at first

analysis (1 hour) was already essentially that ultimately attained at 24 hours, while in the case of mold hydrolysis, reducing power development was much more gradual. For this reason, the Lintner determination (30 minute hydrolysis), so important for malt evaluation, lacks significance when applied to mold culture filtrates. A culture filtrate of *Aspergillus niger* NRRL 330 which had 4.0 alpha-amylase units per ml. and a glucogenic activity of 56.7, when used instead of 5% malt infusion, was found to have a Lintner value of only 34° compared to 180° for the malt. Yet, 5.36 proof gallons of alcohol per bushel of

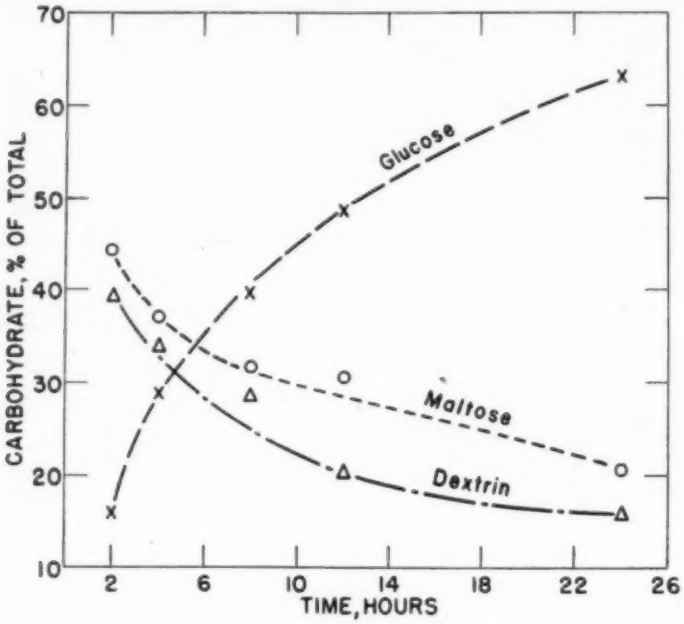

Fig. 3. Starch hydrolysis with *Aspergillus alliaceus* NRRL 315 (at 55°C.) culture filtrate having 16.5 glucogenic activity and 17.5 alpha-amylase units per ml.

corn was obtained through use of this filtrate compared to 5.10 for the malt.

These observations, indicating apparent quantitative production of glucose from starch by potent mold culture filtrates, led to the more detailed hydrolysis investigations that follow.

Composition of Starch Hydrolyzates. The final reducing power developed by most of the mold culture filtrates in the preceding experiments was higher than that developed by malt. Since the latter is known to produce mainly maltose, this observation suggested that substantial quantities of glucose as well as maltose were being produced during starch hydrolysis by molds, and that the total reducing power alone would not be a true index of the amount of fermentable sugars

formed. Therefore, the distribution of glucose, maltose, and unfermentable dextrins as they appeared during hydrolysis of the starch was determined. The starch hydrolysis was carried out as already described for the preceding studies, using the same mold culture filtrates and malt extract.

The course of hydrolysis of 2% soluble starch by a culture filtrate of

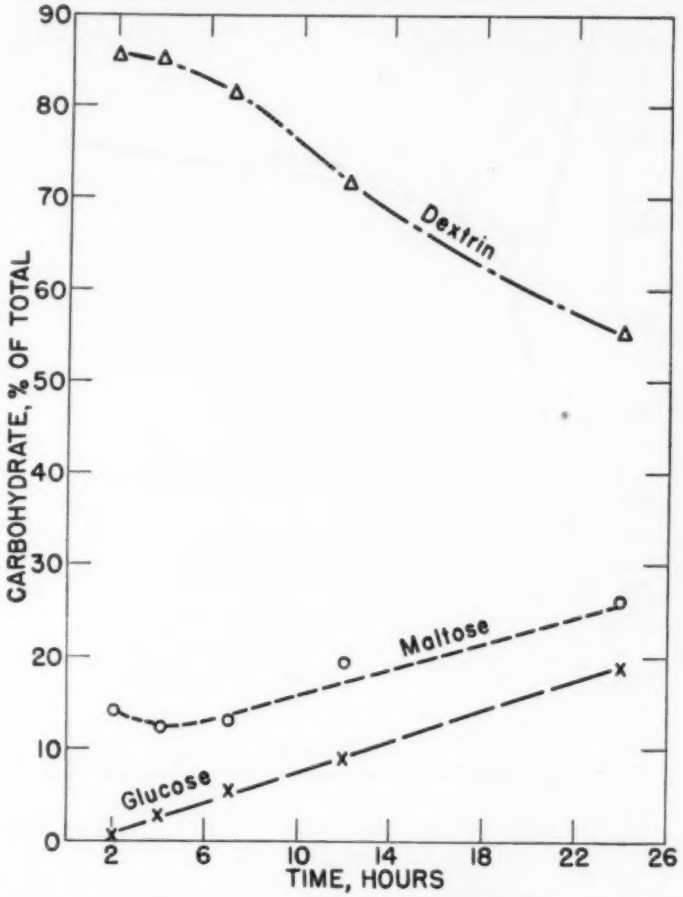

Fig. 4. Starch hydrolysis with *Aspergillus foetidus* NRRL 341 (at 55°C.) culture filtrate having 4.7 glucogenic activity and 3.2 alpha-amylase units per ml.

Aspergillus niger NRRL 330 is presented in Fig. 2. This filtrate had an intermediate alpha-amylase content, high glucogenic activity, and caused rapid development of reducing power (Fig. 1). The hydrolyzate, after 24 hours, was composed of 94% glucose, 2% maltose, and only 4% unfermentable dextrins. The hydrolysis of the starch to glucose was very rapid, over 50% of the hydrolyzate being glucose after 2 hours of incubation, when the first sample was taken.

The culture filtrate from *Aspergillus alliaceus* NRRL 315, which showed only intermediate glucogenic activity but high alpha-amylase potency, gave a lower rate of saccharification (Fig. 3) than did filtrates with high glucogenic activity. The carbohydrates at 24 hours consisted of 63% glucose, 21% maltose, and 16% unfermentable dextrins.

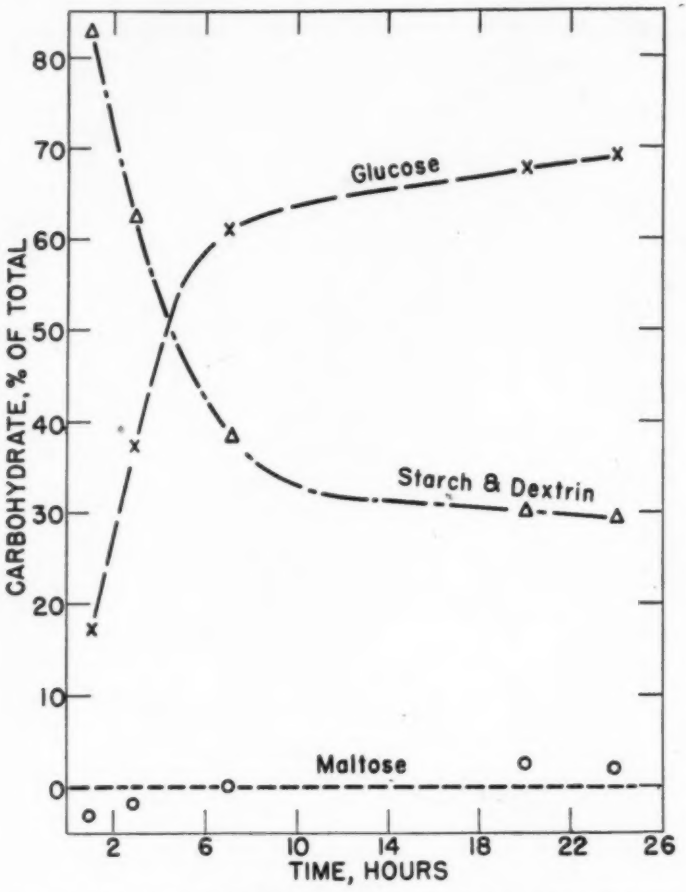

Fig. 5. Starch hydrolysis with *Rhizopus* sp. "Boulard" NRRL 1891 (at 55°C.) culture filtrate having 17.4 glucogenic activity and a trace of alpha-amylase per ml.

It is notable that a high production of maltose occurred in the initial stages of hydrolysis, presumably because of the high alpha-amylase activity of the culture filtrate. The dextrin content as well as the 45% maltose in the 2-hour hydrolyzate gradually decreased as hydrolysis progressed.

The course of starch hydrolysis with the culture filtrate from *Aspergillus foetidus* NRRL 341 is illustrated in Fig. 4. This filtrate

had low glucogenic activity and intermediate alpha-amylase activity. Production of glucose was slow, with about 55% dextrin remaining after 24 hours hydrolysis. From the shape of the curves it appears that the dextrin is gradually degraded and that both maltose and glucose formation followed the dextrinizing reaction.

The culture filtrate from *Rhizopus* sp. "Boulard" NRRL 1891 (the

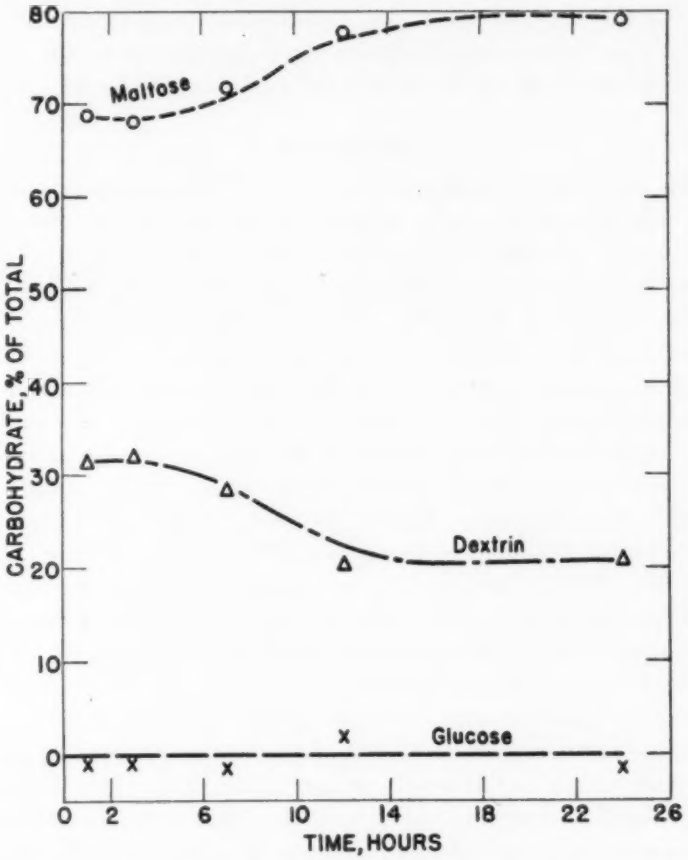

Fig. 6. Starch hydrolysis with 10% extract of 180° Lintner malt (at 55°C.) that assayed 24.0 alpha-amylase units per gram.

strain used in the Amylo process for alcohol production) was of particular interest. This filtrate contained intermediate glucogenic activity but almost no alpha-amylase activity. It caused fairly rapid liberation of glucose with no apparent production of maltose, as shown in Fig. 5. The blue starch-iodine reaction persisted for more than 24 hours, indicating that the dextrin value also included starch which had been hydrolyzed slightly, if at all. At 24 hours the hydro-

lyzate contained 70% glucose, 30% dextrin and starch, and, within experimental error, no maltose. The glucogenic enzyme system in this instance apparently attacked the starch directly to produce glucose.

For comparative purposes, the results of hydrolyzing starch with a 10% extract of barley malt are given in Fig. 6. Malt caused the production mainly of maltose with no glucose, and left 21% unfermentable dextrins in the 24-hour hydrolyzate. The absence of glucose confirmed our finding that no glucogenic activity was present. It should be noted that most of the hydrolysis was completed in the first two hours, when 68% of the starch had been converted to maltose.

Discussion

These experiments indicate that the diastatic system of some mold culture filtrates is primarily composed of a liquefying-dextrinizing enzyme and a saccharifying enzyme system. Thus starch hydrolysis by these fungal preparations is a dual type of saccharification process similar to hydrolysis by malt. Although the action of fungal alpha-amylase is similar to that of malt alpha-amylase, the saccharifying glucogenic enzyme system which liberates glucose differs markedly from saccharifying malt beta-amylase which produces maltose.

In fungal enzyme preparations, alpha-amylase serves to liquefy the starch paste and to rupture the starch molecule with the production of dextrins and, eventually, a limited quantity of maltose. This follows from the fact that fungal preparations with appreciable alpha-amylase activity but of low glucogenic potency caused accumulation of maltose with degradation of dextrin and, in a negative sense, from the fact that no maltose was formed by the *Rhizopus* filtrate in which only a trace of alpha-amylase was present.

The glucogenic enzyme system appears to be active both upon maltose and upon more complex polymers of glucose, since the *Rhizopus* preparation which contains only a trace of alpha-amylase was capable of appreciable degradation of starch to glucose without loss of the blue iodine color. Furthermore, in those cases where a high glucogenic activity is present such as *Aspergillus niger* NRRL 330 (Fig. 2), starch hydrolysis proceeds very rapidly to glucose.

Fungal alpha-amylase and the glucogenic enzyme system apparently supplement each other in the production of fermentable sugars during starch hydrolysis. Thus a comparison of Figs. 3 and 5, in which starch hydrolysis was due to preparations of similar glucogenic activities, shows that there was half as much residual dextrin in the 24-hour hydrolyzate from *Aspergillus alliaceus* NRRL 315, presumably due to the supplementary action of its high alpha-amylase com-

ponent. In Table I the greater alcohol yield associated with *Aspergillus niger* NRRL 337 compared with *Aspergillus wentii* NRRL 377 is also indicative of this supplementary action.

In view of the importance of beta-amylase in the malt diastatic system, its possible presence in the mold enzyme system is of considerable interest. While our information on this subject is as yet quite limited, the low Lintner values of potent fungal preparations indicate the absence of any appreciable beta-amylase activity in mold culture filtrates.

These experiments demonstrate that the evaluation of fungal preparations should include measurement of glucogenic activity as well as alpha-amylase activity, because the rate and completeness of starch hydrolysis depend to a large extent on the activity of the carbohydrase enzyme system that can be measured by its action on maltose.

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EVALUATING THE NUTRITIVE VALUES OF SEVERAL BREADS BY GESTATION-LACTATION PERFORMANCE [1]

ANNABEL BEATY [2] and B. W. FAIRBANKS [3]

ABSTRACT

Six types of bread which had previously been evaluated nutritionally by the rat-growth method were re-evaluated by using the gestation-lactation period as the test period. The females were fed the breads supplemented with 0.1 ml. U.S.P. cod-liver oil and following the growth period they were fed a further addition of 0.1 mg. of alpha tocopherol. Three litters were produced on the diets and statistical analysis revealed that the performance of the third litter gave the most reliable data for evaluation.

Nonfat milk bread was nutritionally superior to water bread, whole wheat bread, and whole wheat nonfat milk bread as determined by statistical analysis.

The differences in the results between enriched water bread and water bread were not statistically significant.

Whole wheat bread was decidedly inferior to all other types of bread with the exception of whole wheat nonfat milk bread which was not significantly superior to it.

The nutritive values measured by gestation-lactation performance were not similar to those obtained by growth studies. The former is a more critical period nutritionally than the growth period, and its use is a more effective means of evaluating diets. In evaluating diets or supplements the period in the life cycle used for testing should be indicated, and differences obtained in one period of the life cycle should not be assumed as applying to another period.

In evaluating the over-all nutritive value of diets or in assaying the effects of single nutritive adjuvants to basal diets, the short time rat-growth method has been extensively employed. If some other period in the life cycle of the rat were used, the results obtained might not be similar to those obtained during growth. Moreover, experimental work in other periods might indicate a period that was even superior to the growth period in ascertaining the comparative nutritive values between diets or supplements under investigation. The literature offers some support to the speculation that the gestation-lactation period might be more critical nutritionally than the growth period. An opportunity presented itself to work with female rats that had been used in a growth study, starting when they weighed approximately 40 g. and continuing for 8 weeks. These females were continued on the same diets and were on test during three successive gestation-lactation periods.

[1] Manuscript received January 2, 1948.
[2] Kraft Foods Company, Chicago, Illinois.
[3] American Dry Milk Institute, Inc., Chicago, Illinois.

In the rat-growth studies grams of gain or grams of food required for a gram of gain have been taken, most generally, alone or in combination, to measure the differences obtained on one or more diets. At times, these have been supplemented by carcass analysis, special determinations, such as hemoglobin and ash content of the bones, and in some instances differences in palatability have been ruled out by equalizing the food intake or by feeding for equal gains. A rather extensive bibliography is presented to support the contention that the gestation-lactation period has possibilities for measuring over-all nutritional effects. It may be a critical period nutritionally, and it affords several observations that lend themselves to measurements. In gestation-lactation there are for observation conception or failure to conceive, the course of pregnancy, gain or loss in weight of the female during pregnancy, the number and individual weights of the young at birth, the number still-born or alive, mortality of the young during lactation which may be recorded in time intervals, the gain or loss in weight of dam, and the number and individual weights of the animals weaned.

In swine nutrition literature there are several papers reporting the inadequacies of experimental rations for normal gestation and lactation. These have been reviewed by Krider, Fairbanks, and Carroll (15) and Fairbanks, Krider, and Carroll (6). Pastures reduced pig mortality in the investigation of Aubel, Hughes, and Leinhardt (2) and Hogan and Johnson (13). Asdell and Willman (1) report the mortality of pigs was twice as high in spring farrowings as in fall farrowings, and it is logical to assume that nutrition during gestation was exerting its influence along with differences in seasons. The number of pigs farrowed and their weights and strength at birth were used as measures of nutritional adequacy of rations during gestation by Gardner (10) and Freeman (9). Some differences noted in gestation-lactation studies are illustrated by the work of Hogan (12) in which 81% of the pigs were weaned on one feeding regime as compared to 51% on another. Hogan and Johnson (14) showed that mortality of young pigs could be reduced by improved nutrition and report that 76% of the pigs farrowed were weaned on a ration that had been quite unsatisfactory before supplementation. Fairbanks, Krider, and Carroll (6) report pig mortalities ranging from 5% to 93% which they attributed to differences in the nutritive value of the gestation-lactation rations. The percentages of pigs weaned varied from 7% to 83% due to differences in the levels of nutrition during gestation and lactation.

Hart, McCollum, Steenbock, and Humphrey (11) indicated that the rations fed pregnant heifers profoundly affected the viability and vigor of their calves.

In human nutrition, the paper of Burke, Beal, Kirkwood, and Stuart (3) is of interest. Their work, including 216 cases, showed the influence of the diet of the mother during gestation on the condition of the infants. Those mothers with diets rated as good or excellent delivered babies of which 42% had pediatric ratings of superior and 55% one or two minor defects. These are to be contrasted with the babies from mothers receiving diets during gestation which were rated as poor or very poor. Only 2.5% of the babies were excellent, 2.5% had one minor defect, 2.8% were in fair or poor condition, while 67% were considered to be in very poor condition. Burke, Harding, and Stuart (4) found a significant relationship between the protein content of the gestation diet and the birth length, birth weight, and the pediatric ratings of the babies.

Wright and Haag (23) working with rats found that l-cystine promoted lactation when added to diets in which the proteins were furnished by alfalfa leaf meal. Normal reproduction and improved lactation in rats were obtained by supplementing rations that had failed to support normal reproduction and lactation, according to the reports of Ross (18) and Cunha (5). The diets fed to breeding females influenced the incidence of congenital abnormalities in their offspring in the investigations of Warkany, Nelson, and Schraffenberger (22). The most recent paper is that of Spitzer and Phillips (21). On their basal diet, more than 35% of the female rats failed to conceive, resorption and toxemia frequently occurred, and the mortality was high one or two days following parturition. Certain supplementations to the basal ration improved the gestation-lactation results.

Materials and Methods

The Bread Samples. The six different kinds of bread fed during the three gestation-lactation periods were the same as those fed during growth by Riggs, Beaty, and Johnson (17). The bread samples were prepared in the National Dairy Research Laboratories, Baltimore, Maryland, as required. Since the experiment lasted several months it seemed advisable to prepare fresh samples periodically. However, all bread samples were prepared from the same lots of white flour and of whole wheat flour representing the whole wheat berry. The bread formulae used throughout were typical of those formulae employed by commercial bakers in the eastern section of the United States, being described as (1) sponge: 58% flour, 2% yeast, 0.5% bread improver, and 41% water; (2) dough: 42% flour, 4% sugar, 2% salt, 2% malt, 1.5% shortening (hydrogenated vegetable oil), and 27.7% water. The supplements were added at the time the dough was made.

The following types of bread were studied:

1.G. *Water bread.* White flour without supplement.

2.G. *Enriched water bread.* White flour enriched with thiamine, riboflavin, niacin, and iron.

3.G. *Nonfat milk bread.* 6% nonfat dry milk solids based upon the weight of white flour.

4.G. *Enriched nonfat milk bread.* Enriched as in 2G and nonfat milk solids as in 3G.

5.G. *Whole wheat bread.* Whole wheat flour, representing the whole wheat berry.

6.G. *Whole wheat nonfat milk bread.* 6% nonfat dry milk solids based upon the weight of whole wheat flour.

The breads were analyzed for the usual constituents by standard methods of analysis and assayed for the vitamins by recognized chemical and microbiological methods. The attempt was made to enrich the white flour to 10% above the minimum levels for thiamine, riboflavin, niacin, and iron as specified in the new standards for enriched flour (Federal Register, 7). The composition of the breads, Table I, indicates that the vitamin enrichment ingredients are above the minimum requirements for enriched bread as specified in the Federal Register (8) with the exception of thiamine, which assayed 0.92 mg. per pound rather than 1.1 mg. as prescribed.

The Experimental Animals. Females of the Sprague-Dawley strain, weighing from 35 to 50 g., were fed ad libitum the same bread diets as reported by Riggs, Beaty, and Johnson (17) and with essentially the same growth response as indicated by the following results at the end of a growth period of 8 weeks. The average final weights of rats receiving the six bread diets were: water bread, 68.6 g.; enriched water bread, 76.9 g.; nonfat milk bread, 128.0 g.; enriched nonfat milk bread, 111.0 g.; whole wheat bread, 104.4 g.; and whole wheat nonfat milk bread, 153.4 g. The corresponding figures for grams of bread per gram gain were water bread, 10.36 g.; enriched water bread, 8.82 g.; nonfat milk bread, 5.10 g.; enriched nonfat milk bread, 5.41 g.; whole wheat bread, 6.03 g.; and whole wheat nonfat milk bread, 4.52 g. During the growth period, bread, water, and a daily dose of 0.1 ml. U.S.P. cod-liver oil were the only constituents of the diet. Eight female rats of each group were continued for four more weeks before mating, each group receiving its respective diet as previously fed with the addition of 0.1 mg. of alpha tocopherol per rat per day.

Following the 12 weeks' feeding the females were placed in group cages, four females to the cage, and one male rat from the stock colony was introduced into each cage. The males were rotated at the

end of each week so that no one male rat remained with any four females for more than one week. Sufficient males were available so that one group of males could usually be kept on the stock diet for one week and then in the mating cages on the bread diets the following week. The rotating of the males among the breeding cages and the

TABLE I

COMPOSITION OF DRY BREAD CRUMBS

Bread	Description of bread	Moisture	Crude protein	Ether extract	Crude fibre	Ash	Thiamine	Riboflavin	Niacin
		%	%	%	%	%	mg./lb.	mg./lb.	mg./lb.
1G	White water bread	9.4	12.9	3.1	0.4	2.68	0.29	0.54	5.93
2G	White water bread, enriched	9.4	13.0	3.0	0.5	2.70	1.34	1.36	17.42
3G	White water bread plus 6% nonfat dry milk solids	8.7	13.9	2.8	0.5	2.93	0.29	0.90	5.50
4G	White water bread, enriched plus 6% nonfat dry milk solids	8.4	13.9	3.0	0.4	2.81	1.36	1.84	20.99
5G	100% whole wheat bread	10.1	14.5	2.6	1.8	3.19	1.17	0.98	15.53
6G	100% whole wheat bread plus 6% nonfat dry milk solids	9.6	15.6	2.9	1.7	3.35	1.04	1.25	14.83
	Chemical composition of bread (38% moisture) calculated from above analyses								
1G	White water bread	38.0	8.8	2.1	0.3	1.83	0.20	0.37	4.06
2G	White water bread, enriched	38.0	8.9	2.1	0 4	1.85	0.92	0.93	11.92
3G	White water bread plus 6% nonfat dry milk solids	38.0	9.5	1.9	0.3	1.99	0.20	0.61	3.74
4G	White water bread, enriched plus 6% nonfat dry milk solids	38.0	9.4	2.0	0.2	1.90	0.92	1.24	14.20
5G	100% whole wheat bread	38.0	10.0	1.8	1.3	2.20	0.81	0.68	10.72
6G	100% whole wheat bread plus 6% nonfat dry milk solids	38.0	10.7	2.0	1.2	2.30	0.71	0.86	10.18

feeding of the males on the stock diets during alternate weeks were done to reduce to the minimum the influence of variations among males and their nutritional status on the performance of the females during gestation and lactation. The same male rats were used during the three gestation-lactation periods.

The females were weighed three times each week and observed for pregnancy. When the female was obviously pregnant she was placed in an individual cage where she remained until weaning her litter at 25 days following parturition.

When all of the females fed the six breads had completed the first gestation-lactation period, they were returned to the mating cages and

the males were again introduced. The length of time between either the weaning or destruction (death or cannibalism) of the first litter and the time the females were returned to the mating cages depended upon the time of birth of the first litter. The rest period was longer in the case of females on diets of nonfat milk bread and whole wheat bread than for those on diets of water bread and enriched water bread.

Following the weaning or destruction (by death or cannibalism) of the second litter, the females were immediately returned to the mating cages without any rest period. The technique employed for the third litter is preferred for test purposes.

Results

The results during gestation and lactation with the first and third litters only are reported in Table II. The second litters have been omitted, but references will be made to results of the second litter when they have a contribution to make.

Statistical Analysis. The data for the number of rats in each litter, the average birth weight of rats in each litter, and the percentage of

TABLE II

RESULTS DURING GESTATION AND LACTATION
(Ad libitum feeding)

Items compared	Group numbers and diets											
	1G		2G		3G		4G		5G		6G	
	Water bread		Enriched water bread		Nonfat milk bread		Enriched nonfat milk bread		Whole wheat bread		Whole wheat nonfat milk bread	
GESTATION												
	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter
Number of litters born	7[1]	8	8	5	8	8	8	8	6[2]	6	8	8
Number of rats born	50	38	45	33	51	62	53	49	36	50	62	70
Avg. no. rats per litter	7.1	4.8	5.6	6.6	6.4	7.8	6.6	6.1	6.0	8.3	7.8	8.8
Avg. birth wt. grams	5.0	5.3	5.0	5.6	4.8	5.6	5.6	5.7	5.5	5.3	5.6	5.8
LACTATION												
	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter	1st litter	3rd litter
Avg. no. rats weaned per litter	0.9	1.0	0.5	2.4	2.4	4.3	2.8	3.0	1.0	0	3.6	0.6
Avg. weaning wt. (grams)	26.6	18.4	12.5	18.9	18.6	23.0	20.0	26.7	23.0	0	27.5	41.0
Percentage of rats died:												
Birth to 3rd day	58.0	34.2	46.7	39.4	43.1	21.0	30.2	14.3	55.6	72.0	41.9	38.6
3rd Day to 7th day	30.0	28.9	26.7	21.2	17.6	17.7	7.5	16.3	5.6	24.0	11.3	35.7
7th Day to 14th day	0	15.8	11.1	3.0	2.0	6.5	9.4	16.3	8.3	4.0	0	15.7
14th Day to 25th day	0	0	6.7	0	0	0	11.3	4.1	8.3	0	0	2.9
Percentage of rats weaned	12.0	21.1	8.9	36.4	37.3	54.8	41.5	49.0	22.2	0	46.8	7.1

[1] One female failed to conceive but produced two subsequent litters.
[2] Two females failed to conceive at any time.

rats in each litter reaching the weaning stage have been submitted to statistical analysis. The first two series of data (rats per litter and average birth weights) yield arithmetic averages to which the analysis of variance could be applied as a test of significance. The third series (percentage of rats weaned), being a set of percentage figures, is more properly analyzed by the chi square test, a technique appropriate to percentages.

The chi square values indicated that the data were not homogeneous by litters, except in the case of water bread and possibly with enriched nonfat milk bread. The number of significant differences between the 15 possible combinations between breads taken two at a time increased from the first to the third litter, indicating an accumulative effect in nutritive value of the six breads.

The statistical study revealed that the greatest number of differences proved to be significant in the third litters and successively increased from the first to the third litters, reflecting the longer time the mothers of the third litters had been on their respective feedings and hence a more extended opportunity for the differences in breads to demonstrate themselves. The data for the third litter are therefore taken as the most significant data in appraising the over-all nutritive value of the six breads under consideration.

Average Number of Rats Born per Litter. The number of live rats born per litter may be an indication of the nutritive value of the diets to permit ovulation, conception, full-term gestation, and parturition beyond the extent that these performances are controlled genetically. In the third litters (Table II), the differences between the water breads and all other breads are significant by analysis of variance in only three cases, nonfat milk bread ($F = 9.89$, df 1 and 14), whole wheat bread ($F = 12.61$, df 1 and 14), and whole wheat nonfat milk bread ($F = 16.25$, df 1 and 14). This analysis of the differences most likely to be significant, namely between water bread and all other breads, indicates that it is unlikely that statistically significant differences can be established by the other types of bread or by order of litters.

In the planning of this preliminary long-time experiment covering three gestation-lactation periods it was decided not to equalize the litters at birth, as the size of the litters and the ability of the females to wean the pups born were considered a part of the data desired. In future and more refined experiments designed to explain differences reported in this trial, it might be advisable to consider equalization of litters.

Average Birth Weights. It is difficult to correlate the average birth weight with the nutritional adequacy of the diet since this figure is influenced by the number of rats born. The results of the first and

third litters, expressed in grams, are enumerated in Table II. By the analysis of variance the differences in the third litter between water bread and the other breads are not significant. In fact an analysis between the differences most likely to be significant, namely water bread and the other breads, indicates only one significant difference of the 15 comparisons and that was between water bread and whole wheat nonfat milk bread in the first litter ($F = 7.47$, df 1 and 14). It is unlikely that any other statistical difference can be established by types of bread and order of litters.

Percentage of Rats Weaned. The most significant data for evaluating the over-all nutritive values of the six breads fed seem to be in the data of percentage of rats weaned in each litter (see statistical analysis) of the third gestation-lactation period (Table II). The total percentage of rats weaned for the six breads are: nonfat milk bread, 54.8; enriched nonfat milk bread, 49.0; enriched water bread, 36.4; water bread, 21.1; whole wheat nonfat milk bread, 7.1; and whole wheat bread, 0.

The females on the water bread diet weaned a significantly lower percentage of rats than the females receiving nonfat milk bread ($P \leqq 0.01$) and enriched nonfat milk bread ($P \leqq 0.01$) and a significantly higher percentage of rats than those receiving whole wheat bread ($P \leqq 0.01$) and whole wheat nonfat milk bread ($P \geqq 0.02$).

The females fed enriched water bread weaned a significantly higher percentage of rats than the females receiving whole wheat bread ($P \leqq 0.01$) and whole wheat nonfat milk bread ($P \leqq 0.01$).

The females receiving nonfat milk bread weaned a significantly higher percentage of rats than those fed water bread ($P \leqq 0.01$), whole wheat bread ($P \leqq 0.01$), and whole wheat nonfat milk bread ($P \leqq 0.01$).

The females on the enriched nonfat milk bread weaned a significantly higher percentage of rats than those on water bread ($P \leqq 0.01$), whole wheat bread ($P \leqq 0.01$), and whole wheat nonfat milk bread ($P \leqq 0.01$).

The females on whole wheat bread weaned a significantly lower percentage of rats than those on all other breads with the exception of whole wheat nonfat milk bread in which the difference was statistically insignificant.

All other comparisons were not statistically significant.

At the time of mating for the first litter there was an extreme difference of 90 g. in the average weight of the rats (water bread, 95 g.; whole wheat nonfat milk bread, 185 g.). This extreme difference had been reduced to 47 g. when the females were mated for the third litter (water bread, 173 g.; whole wheat nonfat milk bread, 220 g.). Body size as

indicated by body weight might be expected to influence performance during gestation-lactation. Yet, the smaller rats on white water bread were able to wean a significantly larger number of young of the third litter than the larger females receiving whole wheat nonfat milk bread or whole wheat bread (184 g.). This accomplishment of the rats on water bread is further emphasized by the fact that they produced significantly smaller litters than those receiving whole wheat nonfat milk bread and whole wheat bread.

Discussion

Riggs, Beaty, and Johnson (17) in a growth study with rats concluded that "the addition of 6% nonfat dry milk solids improves the nutritive value of water bread, enriched water bread, and whole wheat bread." The gestation-lactation results indicate the nutritional superiority of the nonfat milk bread over water bread, whole wheat bread, and whole wheat nonfat milk bread.

The growth studies supported the conclusion that "enrichment at the new levels caused a slight improvement in the nutritive value of water bread which can be observed by paired-feeding for equal gain." The differences between these two breads were not statistically significant in the gestation-lactation studied (ad libitum feeding).

The most surprising results obtained during the gestation-lactation periods were with whole wheat bread and whole wheat nonfat milk bread. Riggs, Beaty, and Johnson (17) concluded from their growth studies, "Water bread supplemented with 6% nonfat dry milk solids and enriched to the levels of the new standards is equivalent in nutritive value to whole wheat bread of average composition as measured by grams of solids required to produce a one-gram gain and the chemical composition of the carcasses of the experimental animals." The results obtained during gestation-lactation clearly indicated that whole wheat bread was decidedly inferior to all other types of bread with the exception of whole wheat nonfat milk bread which was not significantly superior to it.

The results obtained with whole wheat bread during gestation and lactation are deserving of special comment. In the first litters the females weaned only 22.2% of the rats born, and this figure was reduced to 7.5% in the second litter, while the females were not successful in weaning a single rat from the third litters. In both the second and third litters, the females receiving whole wheat bread weaned the lowest percentage of rats. In the second litter, this percentage was significantly lower than enriched water bread ($P \leqq 0.01$), enriched nonfat milk bread ($P \leqq 0.01$), and whole wheat nonfat milk bread ($P \leqq 0.01$). In the third litters, the percentage of rats weaned on the whole wheat

bread diet was significantly lower than any other bread except whole wheat nonfat milk bread in which the difference was not significant. The fertility of the females on the whole wheat breads was not adversely affected.

The experimental technique employed in this initial work is not capable of explaining in terms of nutrition why observed differences occur. At present only the differences are of interest, and further experiments which are better controlled and more refined in procedures will be conducted in an effort to explain the reported observations. Mitchell, Hamilton, and Shields (16) concluded, "There is something in whole wheat, as compared with patent white flour, that impairs calcium utilization." Riggs, Beaty, and Johnson (17) observed that "whole wheat breads vary in nutritive value depending upon the type of whole wheat flour used."

The effect of the addition of nonfat dry milk solids to whole wheat flour on percentage of survivals is noted in the results presented in Table II. The females weaned 46.8% of the pups of the first litter, 48.3% in the second litter, and only 7.1% in the third litter. The addition of nonfat dry milk solids improved the results materially with the first and second litters, but the milk solids, in the amounts used, were not able to maintain a high percentage of pups weaned in the third litter.

These results are not necessarily at variance with the work reported from Sherman's laboratory if differences in the amounts of milk solids fed are considered. Diet A used by Sherman was made up of one-sixth dry whole milk and five-sixths whole wheat plus 2% of sodium chloride based on the weight of the wheat. Diet B consisted of one-third dry whole milk and two-thirds whole wheat plus 2% of sodium chloride based on the weight of the wheat. Sherman and Quinn (20) reported that rats had been maintained for 14 generations on Diet A and for 17 generations on Diet B. The diet containing the higher proportion of dry whole milk produced faster growth and the females reproduced at an earlier age, continued fertile for a longer time, produced larger litters, and raised a greater number of young to weaning age. When the rat colony was in its thirty-fourth generation, Sherman (19) reported that on Diet B, infant mortality had been less and longevity had been increased by 10%. The improvements noted were attributed to the additional amounts of dry whole milk in Diet B.

Mitchell, Hamilton, and Shields (16) stated, "The nutritive deficiencies of patent white flour, and of bread made from it with no nutritive supplements, have been so widely publicized as to need no further comment. However, the conception thus impressed on the public mind that bread is deficient in the essential nutrients would seem

to apply more to the bread formula used many years ago than to modern commercial bread." With these statements in mind it is interesting to note the results obtained with bread made from patent white flour fed continuously during growth and three gestation-lactation periods. The eight females produced 38 rats in the third litter averaging 4.8 rats per litter and an average birth weight of 5.3 g. per rat. The females of this group took much longer to conceive their first litters than all other groups as indicated by an average of 74 days from the time the male was put in the cage until the litter was born. This difference was markedly reduced for the second litter and had disappeared for the third litter. Based on the percentage of rats weaned from the third litters, water bread is nutritionally equal to enriched water bread ($P \geqq 0.05$) and significantly superior to both whole wheat bread ($P \leqq 0.01$) and whole wheat nonfat milk bread ($P \geqq 0.02$).

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STARCH GELATINIZATION STUDIES. II. A METHOD FOR SHOWING THE STAGES IN SWELLING OF STARCH DURING HEATING IN THE AMYLOGRAPH [1]

L. B. CROSSLAND and H. H. FAVOR [2]

ABSTRACT

The stages of swelling occurring during gelatinization of certain starches may be shown by viscosity measurements in the amylograph if a viscous water-binding dispersion medium with proper temperature-viscosity characteristics is employed. This fact is demonstrated herein by utilization of sodium alginate and high viscosity type carboxymethyl cellulose. Starches studied include those of corn, wheat, potato, waxy maize, tapioca, and wrinkled pea.

The gelatinization of certain starches in water with heat takes place in definite stages. Katz referred to the stages as first and second order in connection with the gelatinization of wheat starch (4). An indication by volumetric measurements that these stages are related to changes in size of the granules also demonstrated that the size of granules of certain starches increases by steps with increasing temperature (5). Curves quite similar in nature were produced by study of light transmission during heating of some of the same starches (2). The light transmission studies indicated that the first step in the gelatinization of wheat starch occurs between the temperature of 55°C. and 70°C. and that the greatest effects with rising temperature occur in the second step.

The amylograph as used in cereal laboratories has shown indica-

[1] Manuscript received December 1, 1947.
[2] R. T. Vanderbilt Company, Inc., E. Norwalk, Connecticut.

tions of the effects of varied rates of swelling in different temperature ranges. However, the curves produced have not adequately shown the swelling that occurs at lower temperatures, particularly for those starches where the steps occur at widely separated temperatures.

It has been shown that as the concentration of wheat starch in a suspension gelatinized in the amylograph is raised, the first indications of swelling of starch are apparent at lower temperatures (1). When, for example, 7% wheat starch dispersion is gelatinized in water, no indications of swelling are apparent to approximately 86°C. From 86°C. to a peak at 93°C. the viscosity increases rapidly, thereafter decreasing due to mechanical action. When a 10% wheat starch dispersion is gelatinized, the first indications of swelling are evident at 71°C. The peak viscosity occurs at a slightly lower temperature than for the 7% concentration, but the more rapid decrease in viscosity after the peak is evidence of greater mechanical breakdown of granules. At the 10% concentration, swelling of granules is effectively shown over the range from 71°C. to about 93°C. or a span of 22°C. Any further increase of starch concentration would result in a viscosity greater than that measurable by the machine. Up to a 10% concentration it is not possible to produce strong evidence of two stages of gelatinization for wheat starch if water is used as the dispersion medium. It appears that some modification of the technique other than that of changing levels of wheat starch must be employed if the stages of gelatinization are to be studied by viscosity measurements.

Employment of a viscous dispersion medium in place of water in gelatinization studies should afford several advantages. Primarily, it should magnify the viscosity effect of small changes in size of granules. The low starch concentration required to give evidence of swelling would reduce the friction between granules and consequent mechanical breakdown when the swelling is at a maximum.

The purpose of this article is to demonstrate how the accepted technique of amylograph starch gelatinization studies may be modified to show more adequately the swelling that occurs during heating of some starches.

Materials and Methods

Description, function, and uses of the Brabender Amylograph were published by Anker and Geddes (1) and by Brown and Harrel (3). The essential difference between the technique described herein and that used in other starch viscosity studies with the amylograph is that a viscous, water-binding dispersion medium of low solids content is utilized in place of water.

Dispersions of sodium alginate and carboxymethylcellulose were

made by adding weighed quantities of the powders to warm distilled water while agitated in a Waring Blendor. The dispersion was weighed into the amylograph bowl and the temperature held at 40°C. while the machine was operated until the viscosity curve became flat. A weighed amount of starch was added and dispersed as completely as possible with a spatula. The mixture was afterward held at 40°C. and stirred for 5 minutes to disperse the starch further and bring the viscosity to constant value. The temperature was then increased at the rate of 1.5°C. per minute to 100°C.

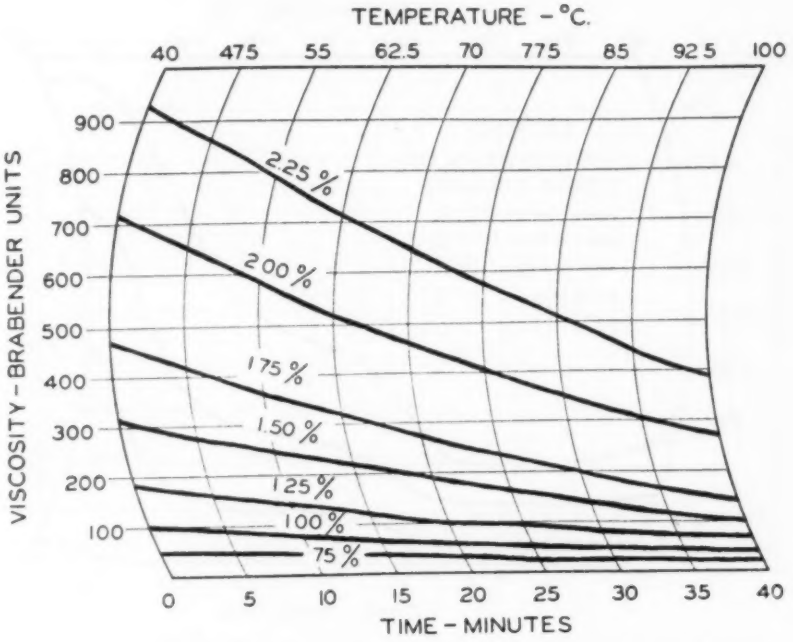

Fig. 1. Relation of viscosity to temperature of various concentrations of sodium alginate from 0.75% to 2.25% solids. Samples of 350 g. were used in all cases.

The lowest concentration used in preparation of curves in this work is 0.75% of sodium alginate; the highest concentration is not known as it is a function of the water taken from the dispersion medium by the starch during gelatinization. It is quite improbable that the effective concentration of sodium alginate in these experiments ever reaches a concentration of 2.25% based on the water not bound by the starch.

Temperature Effect on Viscosity of Dispersion Medium. The effect of temperature on various concentrations of sodium alginate was investigated within the range of concentrations of 0.75% to 2.25% solids; 350 g. of dispersion were used in each case. Fig. 1 shows that

for all concentrations of sodium alginate the viscosity changes at reasonably uniform rates and that in no case is there an increase of viscosity with increase of temperature.

Effect of Concentration of Dispersing Medium. Portions of 25 g. of wheat starch were gelatinized in 350 g. each of water and dispersions of alginate at concentrations of 0.75%, 1.00%, and 1.25% solids.

As is shown by Fig. 2, the contour of the curves resulting from the gelatinization of starch in a viscous-dispersing medium is influenced by the solids content of the dispersing medium. This may reasonably

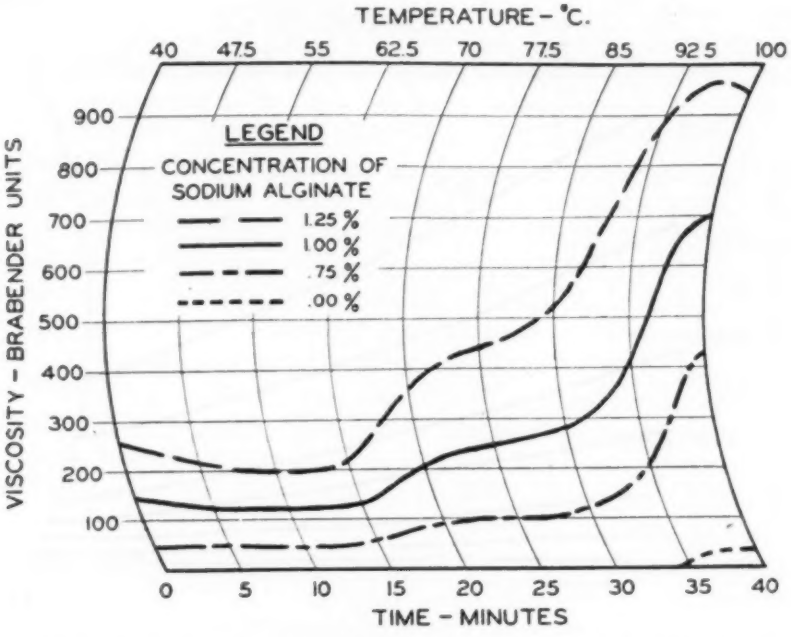

Fig. 2. Effect of the viscosity of the dispersing medium on the contours of the gelatinization curve of wheat starch. Starch samples of 25 g. were dispersed in 350 g. of medium in each case.

be expected because of the complex relations of viscosity to concentration of dispersions.

It will be noted that the first effects of swelling of the starch are indicated at a temperature of 90°C. when the dispersing medium is distilled water only. However, in the starch-alginate-water system the first indications are shown at about 55°C. and change little with wide variations in concentration of sodium alginate.

Effect of Starch Concentration. As the dispersing medium for gelatinization of various levels of wheat starch 350 g. of 1.25% sodium alginate dispersion were used. The levels were 0, 12.5, 25, and 50 g. of wheat starch.

It is shown in Fig. 3 that as the level of starch gelatinized in a given dispersion medium is increased, the temperature of initial swelling will be more sharply defined. It is shown, also, that the temperature of initial indication of swelling remains fairly constant as starch levels vary in contrast to the situation when water alone is used as the dispersing medium (1).

Varied levels of starch produce greater effects in contours of the curves than does variation of concentration of the medium, as may be seen by comparison of Figs. 2 and 3. While one curve produced by

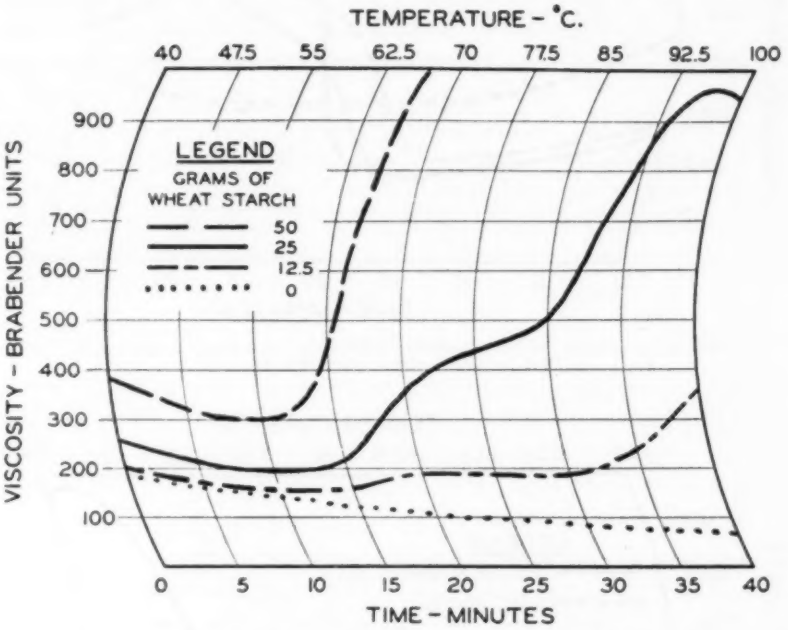

Fig. 3. Effect of various levels of wheat starch on the nature of the curve when gelatinized in 350 g. of 1.25% sodium alginate dispersion.

proper concentration of starch and dispersion medium may prove the existence of stages during gelatinization, it is necessary to examine families of curves to conclude that stages do not exist. Comparison with curves obtained by light transmission studies (2) discloses close agreement in temperatures where first effects of heating are evident.

Differences in Swelling of Various Starches. Fig. 4 shows the curves produced by several types of starches gelatinized in 350 g. of 1.25% sodium alginate dispersion. The quantity of starch used was selected to give best indications of existence of stages for those found to swell by stages. Various levels of tapioca and waxy maize up to 20 g. with concentrations of sodium alginate from 0.75% to 1.25%

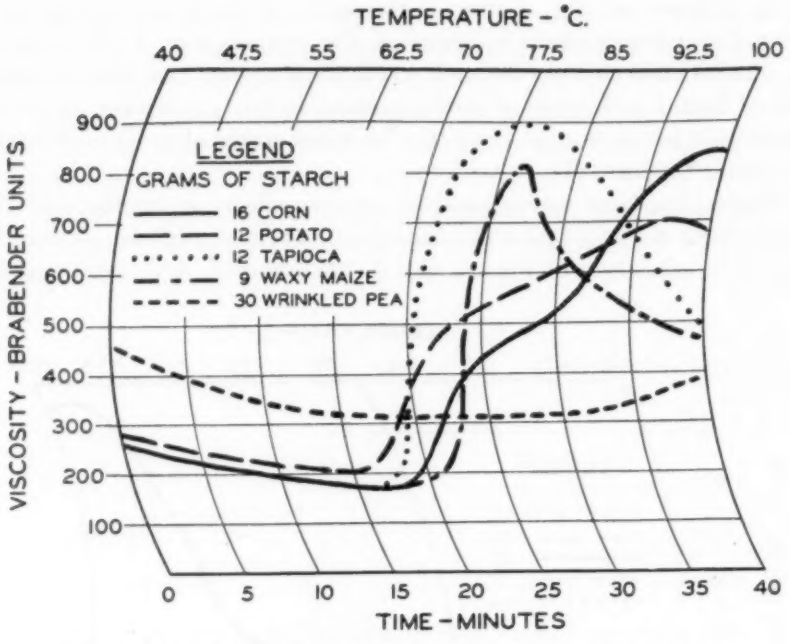

Fig. 4. The nature of curves produced by various types of starch gelatinized in 350 g. of 1.25% sodium alginate dispersion.

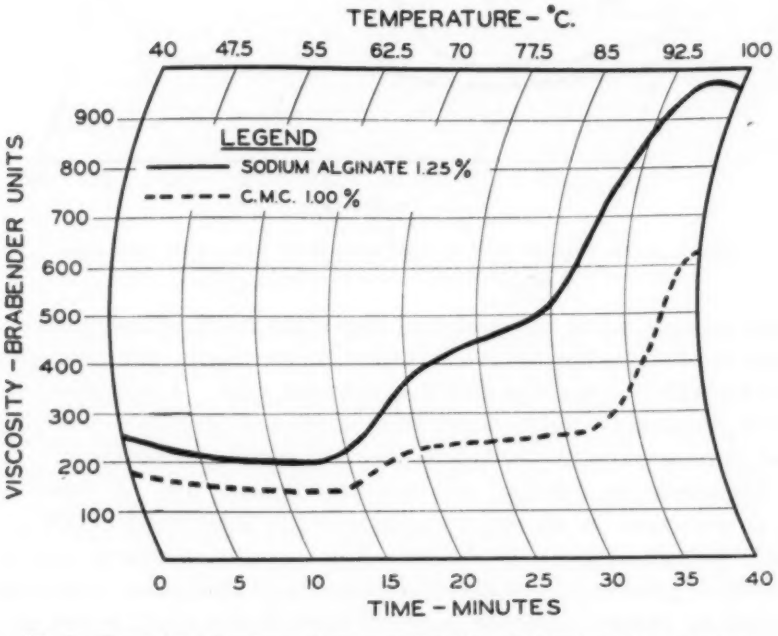

Fig. 5. Comparison of contours of curves produced by gelatinization of 25 g. of wheat starch in solutions of high-viscosity carboxymethylcellulose and sodium alginate.

failed to reveal the existence of stages. For those starches exhibiting only one stage of gelatinization, curves were selected in which the peak viscosity was about 850 Brabender Units.

The curves for corn and potato show the existence of marked differences in the rate of swelling of starch granules in different temperature ranges. The curve for wrinkled pea starch by its comparatively high viscosity indicates that a large amount of water is absorbed initially. The slope of the curve remains fairly constant to a temperature of 55°C. when it goes through zero and slowly becomes greater, indicating that swelling of wrinkled pea starch does not occur at critical temperatures above 40°C. A test with initial temperature at 25°C. also failed to disclose a critical temperature.

Effect of Composition of Dispersion Medium. Fig. 5 shows the similarity between contours of curves produced by gelatinization of 25 g. of wheat starch in 350 g. of 1% solution of high viscosity type carboxymethylcellulose and a 1.25% dispersion of sodium alginate, suggesting that the viscosity changes with temperature shown in the curves of Figs. 2, 3, and 4 are not the result of a specific reaction between the starch and solids of the dispersion medium.

Discussion

It is shown in Fig. 2 that at any temperature the slope is greater as the concentration of the dispersion medium is greater. This relation holds except in the cases where the viscosity exceeds 400 Brabender Units. This exception may be due to either, or a combination, of two effects.

1. High shearing forces causing disintegration of the granules.
2. Increased resistance by the more concentrated dispersion medium to removal of water by the starch, thereby inhibiting maximum swelling of starch granules.

Strong evidence of the action of the first effect is given by the fact that at the highest concentration of dispersion medium a peak viscosity is reached followed by a drop.

Acknowledgment

To Thomas J. Schoch of Corn Products Refining Company, Argo, Illinois, through whose courtesy the sample of wrinkled pea starch was furnished for this study, indebtedness is expressed.

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COMPARATIVE STUDY OF THE EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN ON UNSALTED AND SALTED DOUGHS[1]

ROSA STERN[2]

ABSTRACT

The effects on doughs of cysteine hydrochloride and papain at two levels of addition were found to be quantitatively different. The amount of reducing matter in the washings from doughs containing papain was practically the same as that in the washings from control doughs, whereas, when cysteine hydrochloride was added to a dough, increasing amounts resulted in increasing quantities of reducing matter in the washings. It is concluded from these findings that the mechanism of cysteine action is different from that of papain action.

Neither cysteine hydrochloride nor papain affected the cystine content of the gluten. Thus, these agents behaved like the thiol compounds and protease present in wheat germ extract.

Washings from unyeasted doughs containing cysteine hydrochloride gave a positive reaction with sodium nitroprusside even after 5 hours dough time; whereas, in the washings from yeasted doughs, the reaction was negative even after 2 hours dough time.

Presence of sodium chloride in dough and washing water led to a decrease of the cystine content of the gluten and of the reducing matter in the washings. Fermentation of salted doughs caused no further decrease in the cystine content of the gluten.

Presence of sodium chloride in dough and wash water checked the gluten breakdown caused by cysteine hydrochloride, and partly so, that due to papain.

The earlier finding of the author that fermentation causes a decrease of the cystine content of the gluten in unsalted doughs was confirmed.

The literature up to 1943 dealing with the effects on dough of oxidizers, reducers, and proteolytic enzymes has been reviewed by Shen and Geddes (5) and by Stern (6). Several papers on this subject have since been published and the more pertinent of them are cited throughout this paper where they have a bearing on points under discussion.

The experiments reported below were undertaken to find further

[1] Manuscript received June 3, 1946.
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evidence as to whether thiol compounds function merely as activators of proteolytic enzymes or whether their action is different from that of these enzymes.

In an earlier investigation Stern (6) studied the separate effects of the protease and the thiol groups naturally present in wheat germ extracts. The present experiments deal with the effects of cysteine hydrochloride and papain added to doughs.

Because of the similarity of the problems the methods applied in the earlier work were also used in the present one. The following reasoning motivated the selection of these methods:

1. If the effects of thiol groups are different from those of proteases of the papain type it might be expected that the amount of soluble protein in the treated doughs and the degree of mechanical breakdown of the gluten caused by one agent would be different from those due to the other. Accordingly, the analytical method was to wash gluten from the doughs and determine the nitrogen content of the washings. Loss of gluten in the washing process was regarded as a measure of mechanical breakdown, and was estimated as the difference between the total nitrogen of the flour and the nitrogen found in the solubles and in the coherent part of the gluten. (Loss of gluten = total nitrogen − soluble nitrogen − nitrogen of coherent gluten.)

2. If, as Sullivan and co-workers (7) suggested, thiol groups react with disulfide groups of gluten, the gluten washed from a dough treated with thiol compounds should contain less cystine than that from a blank dough, because part at least of the disulfide linkages originally present would have undergone chemical change. It should also contain less cystine than gluten from a dough treated with papain which, although containing thiol groups, would supply a comparatively insignificant amount of them. Accordingly, cystine determinations were carried out in the hydrolyzates of the coherent part of glutens washed from treated and untreated doughs. Total cystine (in the coherent and the broken-down fractions) was calculated by the following formula:

$$\text{Total cystine} = \text{cystine found}/(100 - \text{loss of gluten nitrogen}).$$

As discussed in the earlier paper, this formula implies that the cystine content of the coherent part of the gluten is the same as that in the broken-down fraction. It follows that the cystine determined in the coherent fraction should bear the same relation to the cystine present, but not determined, in the disintegrated part as the nitrogen contents of these fractions bear to each other. Significant changes in total cystine (compared with the figures applying to gluten from an unyeasted blank dough) would indicate that the treatment caused either

a change in the cystine content of the gluten or a change in the cystine distribution between coherent and disintegrated fractions.

In addition to the determinations discussed above, the reducing matter in the dough solubles was estimated, and tests with sodium nitroprusside were carried out.

The experiments, with a view to practical baking conditions, covered salted as well as unsalted doughs.

Materials and Methods

Materials. The flour used was a commercial straight run flour milled from New Zealand wheat. It contained 15.7% moisture, 0.40% ash, and 8.1% protein, and gave a satisfactory mature loaf when baked according to the A.A.C.C. formula. Some of the experiments were repeated on other flours, but as the results obtained were not basically different they will not be reported here.

The cysteine hydrochloride was a preparation from Hoffmann LaRoche and Company. The papain was a Kahlbaum product; it contained 264 mg. nitrogen per g. and reduced 2.38 ml. $N/100$ iodine solution per g.

Methods. The gluten washing process was carried out by hand, using 10 separate portions of tap water, or of 2% sodium chloride solution. No silk was used.

The gluten recovered as a coherent lump was torn into small pieces and hydrolyzed in approximately 6 N sulfuric acid solution for 18 hours.

Total nitrogen was determined in the flour and in the coherent part of the gluten (by analyzing an aliquot of the unfiltered gluten hydrólyzate). Soluble nitrogen was determined in an aliquot of the filtered combined washings, the volume of which was assumed to be 514 ml. (500 ml. water used for washing, plus 15 ml. dough liquid, plus 3.9 ml. flour moisture, minus 4.7 ml. hydration water of wet gluten). The mechanical loss due to gluten breakdown was found by deducting the nitrogen of coherent gluten plus soluble nitrogen from the total nitrogen and was expressed in per cent of total gluten nitrogen.

Cystine was determined in aliquots of the filtered gluten hydrolyzates. The method of Mirsky and Anson (4) was used, and extraneous reducers were determined according to Lugg (3). In applying the technique of Mirsky and Anson, however, urea was not employed. The values obtained in absence of urea were, on the average, 15% lower than when urea was present, but the trend within each series of experiments was the same. Total cystine was computed by the formula: total cystine = cystine found/(100 − % gluten nitrogen lost) (Stern, 6).

Reducing matter in the washings was determined as follows: As soon as the washing process was finished, the combined washings were thoroughly mixed, and roughly 150 ml. of the suspension were centrifuged. Portions of 100 ml. of the centrifugate were deproteinized with sulfuric acid and sodium tungstate (*Cereal Laboratory Methods*, 1941, p. 100), and again centrifuged. To 100 ml. of this second centrifugate (corresponding to 92.6 ml. of the first one) 5 ml. or, if necessary, 10 ml. of *N* 500 iodine solution were added, and the excess was backtitrated with *N* 500 sodium thiosulfate solution.

The nitroprusside reaction was carried out in the washings obtained from the first 50 ml. portion of water or salt solution used, as the concentration of thiol groups in the combined washings is too low to give a positive reaction. The suspension resulting from washing with this first portion of washing liquid was centrifuged, a few ml. of the centrifugate were saturated with sodium chloride, acidified with acetic acid, three drops of a 5% solution of sodium nitroprusside were added, and the contents of the test tube made slightly alkaline with ammonia. A color change to a purple or purplish hue indicated the presence of thiol groups.

Results

Experiments with Unsalted Doughs Washed with Tap Water. The doughs for the first series of experiments were made from 25 g. of flour mixed with 15 ml. of tap water, and tap water was also used in washing out the gluten. When the doughs were to be fermented, 0.5 g. of yeast was suspended in the dough liquid. Cysteine treatment involved addition of 2.55 mg. cysteine hydrochloride (0.01% on flour basis), and papain treatment involved addition of 7.5 mg. papain (0.03% on flour basis).[3] Dough times of 1, 3, and 5 hours were used, and all doughs were maintained at 27.8°C. Table I shows the results obtained in these experiments.

Unfermented Doughs. The data indicate that only papain caused an appreciable increase in soluble nitrogen with dough time. Gluten losses increased with dough time at similar rates in both cysteine and papain doughs. The cystine content of the gluten was independent of dough time. An analysis of variance showed that neither cysteine hydrochloride nor papain significantly affected the cystine content of gluten. The reducing matter in the deproteinized washings from the cysteine doughs was very much in excess of that in the washings from either blank or papain doughs. The reducing matter content of

[3] The additions of cysteine hydrochloride and papain were chosen so as to cause moderate and approximately equal damage to the gluten on washing, at the lower level of addition. In the baking test, the deterioration due to corresponding additions was much more severe in the papain dough than in the cysteine dough. The concentrations used lie within the limits of additions applied by other workers.

the last two was practically the same. The nitroprusside reaction of the washings from the cysteine doughs was positive after 5 hours dough time.

Fermented Doughs. Soluble nitrogen was higher than in the unfermented series and, in all doughs, increased with dough time. There was, however, no significant difference between the soluble nitrogen in cysteine doughs and in papain doughs at the level of addition employed. The gluten losses were also much larger than in the unfermented series and failed to reveal a difference between treatment

TABLE I

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN AT VARYING DOUGH TIMES ON UNYEASTED AND YEASTED DOUGHS, UNSALTED AND WASHED WITH TAP WATER

(Dough times of 1, 3, and 5 hours at 27.8°C.
Total nitrogen in 25 g. flour = 357 mg.)

Determination	Dough-time hrs.	Without yeast			With yeast		
		control	cysteine	papain	control	cysteine	papain
Nitrogen in coherent gluten, mg.	1	300	289	292	279	261	273
	3	289	273	272	220	152	148
	5	293	269	249	177	103	87
Soluble nitrogen, mg.	1	48	52	50	59	72	65
	3	51	56	56	78	86	84
	5	49	55	60	88	97	96
Lost nitrogen in % of total gluten-nitrogen	1	2.9	5.2	4.9	6.4	8.4	6.5
	3	5.6	9.3	9.6	21.1	43.9	45.8
	5	4.9	10.9	18.5	34.2	60.4	66.7
Total cystine in gluten, mg.	1	44.0	45.0	44.6	43.6	42.7	45.1
	3	44.6	42.1	43.9	40.8	38.7	40.6
	5	43.5	45.1	45.6	40.9	41.9	43.2
Reducing matter, as ml. *N*/500 iodine solution	1	1.7	11.6	1.5	3.4	13.4	2.3
	3	1.7	11.2	1.4	4.2	16.5	4.8
	5	2.8	12.8	2.6	3.7	14.3	3.4

with cysteine and papain. The cystine content of the glutens was not affected by either of the additions. Dough fermentation, however, caused a significant decrease of cystine in the glutens from all the doughs. Both these observations are in keeping with the results obtained in the earlier work on the effects of wheat germ. Reducing matter was generally higher than in the unfermented doughs, and here again the figures for the cysteine doughs were very much higher than those for either the papain or control dough. The nitroprusside reaction in the washings from the cysteine doughs was weak after 1 hour and negative after 2 hours dough time.

In summary, the results contained in Table I indicate that, at the applied level of additions, the only significant difference between cysteine and papain treatments was the content of reducing matter in the respective doughs. The experiments revealed that, independent of any additions, dough fermentation lowered the cystine content of gluten.

A further series of experiments involved additions of cysteine hydrochloride and papain at a level 100% higher than that applied in the first series. Table II gives a comparative summary of the values obtained at both levels of addition.

TABLE II

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN, AT TWO LEVELS OF ADDITION, ON UNYEASTED AND YEASTED DOUGHS, UNSALTED AND WASHED WITH TAP WATER

(Dough time 3 hours at 27.8°C. Total nitrogen in 25 g. flour = 357 mg.)

Determination	Without yeast				With yeast			
	cysteine		papain		cysteine		papain	
	2.55 mg.	5.1 mg.	7.5 mg.	15.0 mg.	2.55 mg.	5.1 mg.	7.5 mg.	15.0 mg.
Nitrogen in coherent gluten, mg.	273	161	272	28	152	143	148	4
Soluble nitrogen, mg.	56	66	56	90	86	93	84	133
Lost nitrogen in % of total gluten-nitrogen	9.3	44.7	9.6	89.5	43.9	45.8	45.8	98.2
Total cystine in gluten, mg.	42.1	46.5	43.9	44.4	38.7	35.2	40.6	38.9
Reducing matter, as ml. *N*/500 iodine solution	11.2	25.1	1.4	1.7	16.5	30.9	4.8	4.5

Unfermented Doughs. Soluble nitrogen as well as gluten losses increased with increasing additions. At the higher level, these increases were much larger in the papain than in the cysteine doughs. Total cystine was again unaffected by either agent.

Reducing matter in the washings from the cysteine doughs increased roughly in proportion to the increased addition, whereas it remained practically constant in the washings from the papain doughs and controls.

Fermented Doughs. Soluble nitrogen increased from the lower to the higher level of addition, much more so in the papain doughs than in the cysteine doughs. Gluten losses on washing were but little in-

fluenced by the increased addition of cysteine hydrochloride, whereas the increased addition of papain caused almost complete breakdown of the gluten. The gluten breakdown caused by cysteine hydrochloride does not seem to proceed beyond a certain limit. This observation is in keeping with the findings of Ford and Maiden (2), Swanson and Andrews (8), and of Balls and Hale (1).

Total cystine did not respond to increased additions of either cysteine hydrochloride or papain. Fermentation again lowered the cystine content of the gluten. Reducing matter in the cysteine doughs, as in the unfermented series, increased roughly in proportion to the addition made.

Experiments with Salted Doughs Washed with Salt Solution. The doughs for this series of experiments were made from 25 g. of flour mixed with 15 ml. of tap water containing 0.5 g. of salt. When the doughs were to be fermented, 0.5 g. of yeast was suspended in the dough. A 2% solution of commercial salt was used for washing out the gluten. The additions were 2.55 mg. of cysteine hydrochloride or 7.5 mg. of papain. The doughs were kept at 27.8°C. for 3 hours.

Table III shows the results obtained in this series.

TABLE III

EFFECTS OF CYSTEINE HYDROCHLORIDE AND PAPAIN ON UNYEASTED AND YEASTED DOUGHS, SALTED AND WASHED WITH A 2% SODIUM CHLORIDE SOLUTION

(Dough time 3 hours at 27.8°C. Total nitrogen in 25 g. flour = 357 mg.)

Determination	Without yeast			With yeast		
	control	cysteine	papain	control	cysteine	papain
Nitrogen in coherent gluten, mg.	281	282	275	283	285	190
Soluble nitrogen, mg.	64	69	70	58	59	76
Lost nitrogen in % of total gluten	4.1	2.1	4.2	5.3	4.4	32.4
Total cystine, mg.	41.9	40.8	38.5	40.8	42.7	41.1
Reducing matter, as ml. *N*/500 iodine solution	0	7.2	0	0	10.9	0

The values for soluble nitrogen and loss of gluten are different from the corresponding values obtained in absence of salt, being higher in the unfermented and lower in the fermented series. A significant increase in soluble nitrogen and loss of gluten occurred only in the fermented dough containing papain. The cystine content of the glutens was unaffected by either cysteine hydrochloride or papain.

Reducing matter disappeared from the washings of all doughs except those containing cysteine hydrochloride.

The nitroprusside test was positive in the unyeasted and negative in the yeasted cysteine doughs.

The use of sodium chloride resulted in lowering the cystine content of the glutens from all doughs, and in eliminating the difference between unyeasted and yeasted doughs which was observed in absence of salt.

Discussion

As previously stated, the objective of these experiments was to secure data which would aid in elucidating the mechanism of the action of cysteine and papain in doughs.

At the lower levels of addition, the effects of cysteine hydrochloride and papain, expressed in terms of soluble nitrogen and gluten breakdown, were approximately equal, but at the higher levels papain produced much more soluble nitrogen and caused a far severer breakdown of the gluten. These observations are in agreement with the findings of Swanson and Andrews (8) who concluded that the mechanism of papain action is different from that of cysteine action.

The figures for soluble reducing matter also indicate that cysteine hydrochloride affects the dough in a different way from papain. Doughs containing either of these agents in amounts causing approximately equal damage yielded washings which differed widely in their contents of reducing matter. The quantity of reducing matter washed from cysteine doughs was a multiple of that present in the washings from papain doughs. The further observation that the reducing matter washed from cysteine doughs was roughly proportional to the amount of cysteine added suggests that the cysteine hydrochloride (or the bulk of it) was released by the washing process, or that some other water-soluble reducing substance was formed. In either case, the linkage between gluten proteins and cysteine must have been a loose one. That disulfide linkages of the gluten were not involved in this linkage is borne out by the fact that the cystine content of the gluten remained unchanged. The gradual improvement which doughs containing thiol compounds undergo on prolonged fermentation would indicate that the bond between thiol compounds and gluten proteins is such that it leaves the thiol group free to be oxidized or otherwise to react. The nitroprusside test showed that thiol groups gradually disappear in a fermenting dough, but this does not result in a decrease of reducing matter. It would appear that the reducing matter washed from unyeasted cysteine doughs was unchanged cysteine hydrochloride, whereas the reducing matter washed from yeasted doughs was a re-

ducing compound deriving from cysteine, perhaps the sulfenic or sulfinic acid.

These conclusions were drawn from values which show a similar trend in both unsalted and salted series. The differences between those two series, involving not only soluble nitrogen and gluten losses, but also reducing matter and cystine content, are probably all attributable to changes in protein solubility in the presence of sodium chloride.

Acknowledgments

The author wishes to record her grateful appreciation of Peggy Aiken's able and untiring help in the analytical part of this investigation. Her thanks are also due to Sheila Boyce for advice and help in the application of statistical methods.

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SUGGESTIONS TO AUTHORS

General. From January 1, 1948, an abstract will be printed at the beginning of each paper instead of a summary at the end, references will be numbered to provide the option of citing by number only, and date of receipt, author's connections, etc., will be shown in footnotes. Except on these points, authors will find the last volume of *Cereal Chemistry* a useful guide to acceptable arrangements and styling of papers. "On Writing Scientific Papers for Cereal Chemistry" (*Trans. Am. Assoc. Cereal Chem.* **6**: 1–22. 1948) amplifies the following notes.

Authors should submit two copies of the manuscript, typed double spaced with wide margins on 8½ by 11 inch white paper, and all original drawings or photographs for figures. If possible, one set of photographs of figures should also be submitted. Originals can then be held to prevent damage, and the photographs can be sent to reviewers.

Titles and Footnotes. Titles should be specific, but should be kept short by deleting unnecessary words. The title footnote shows "Manuscript received . . ." and the name and address of the author's institution. Author footnotes, showing position and connections, are desirable although not obligatory.

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Text. Clarity and conciseness are the prime essentials of a good scientific style. Proper grouping of related information and thoughts within paragraphs, selection of logical sequences for paragraphs and for sentences within paragraphs, and a skillful use of headings and topic sentences are the greatest aids to clarity. Clear phrasing is simplified by writing short sentences, using direct statements and active verbs, and preferring the concrete to the abstract, the specific to the general, and the definite to the vague. Trite circumlocutions and useless modifiers are the main causes of verbosity; they should be removed by repeated editing of drafts.

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Use names, not formulas, for text references to chemical compounds. Use plural verbs with quantities (6.9 g. were). Figures are used before unit abbreviations (3 ml.), and % rather than "per cent" is used following figures. All units are abbreviated and followed by periods, except units of time, which are spelled out. Repeat the degree sign (5°–10° C.). Place 0 before the decimal point for correlation coefficients (r = 0.95). Use * to mark statistics that exceed the 5% level and ** for those that exceed the 1% level; footnotes explaining this convention are no longer required. Type fractions on one line if possible, e.g., A/(B + C). Use lower case for farinograph, mixogram, etc., unless used with a proper name, i.e., Brabender Farinograph. When in doubt about a point that occurs frequently, consult the Style Manual or the Dictionary.

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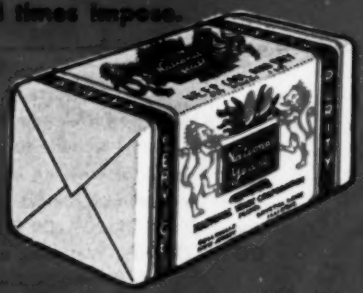

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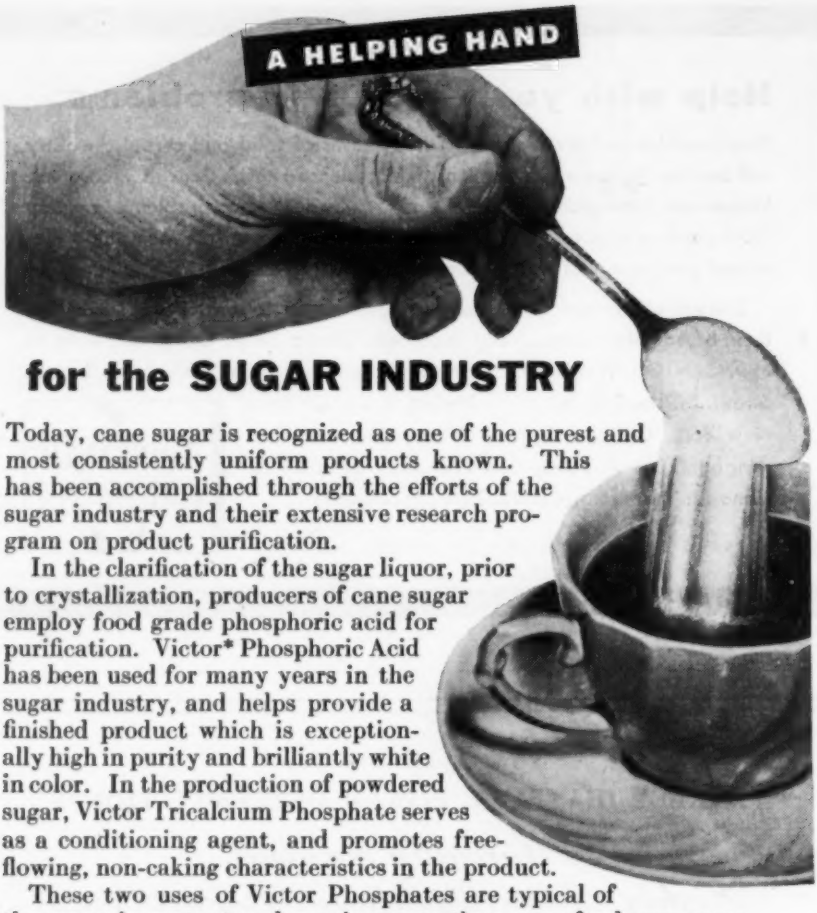

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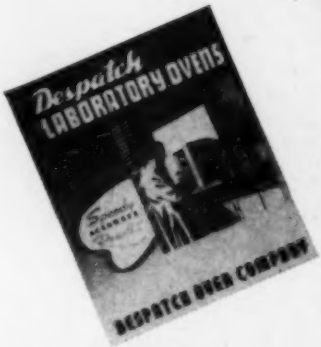

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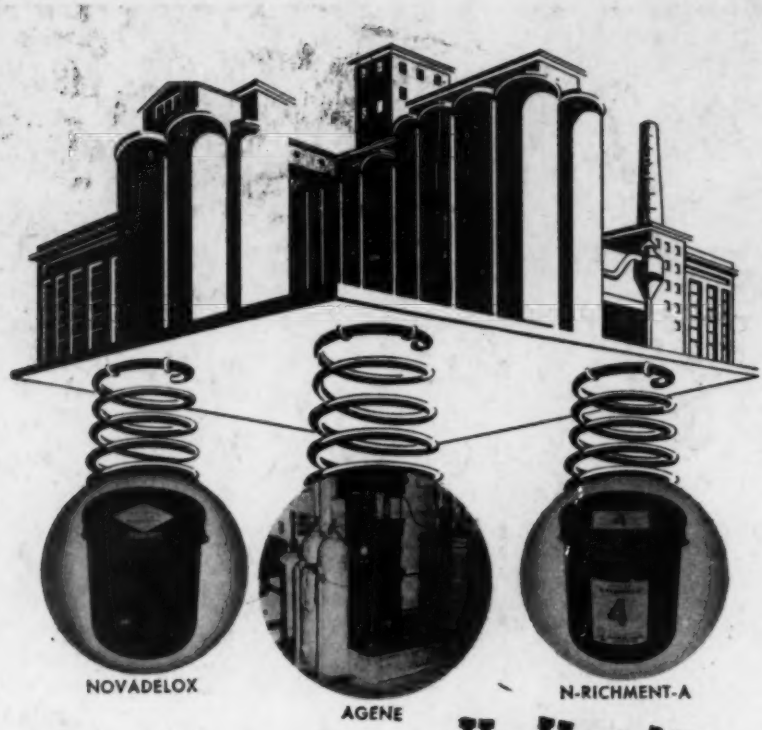

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